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Hans Gerhard Vogel
Editor

Drug Discovery and Evaluation: Pharmacological Assays

3rd Edition

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Drug Discovery and Evaluation: Pharmacological Assays

H. Gerhard Vogel (Ed.)

Drug Discovery and Evaluation: Pharmacological Assays

Third Completely Revised, Updated and Enlarged Edition

With 5 Figures and 66 Tables

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Hans Gerhard Vogel
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Preface to the Third Edition

The third edition of the book *Drug Discovery and Evaluation. Pharmacological Assays* is presented here.

The volume of data has risen considerably compared to the second edition. In particular, a large number of assays have been added. Meanwhile, data on Safety Pharmacology have been removed from this edition. An extended discussion of that important aspect of pharmacology was published in the book *Drug Discovery and Evaluation. Safety and Pharmacokinetic Assays*, edited by H. G. Vogel, F. J. Hock, J. Maas, and D. Mayer, by Springer in 2006.

Several of my colleagues provided essential contributions to this edition. In particular I am indebted to S. A. Mousa for rewriting the chapter Pharmacological Assays in Thrombosis and Hemostasis, S. G. E. Hart, for rewriting the chapter Activity on Urinary Tract, G. Müller for rewriting and amending the chapter Antidiabetic Activity, B. Schultz for amending the chapter Ophthalmologic Activity, and J. Sandow for rewriting the chapter Endocrinology. All chapters have been revised and thoroughly updated as well.

The approach to drug discovery is changing continuously. Decades ago, many drugs were found by serendipity in clinical trials. Most new drugs, however, were found by the classical approach in animal experiments. This approach has the advantage of relatively high predictability, but it also has the disadvantage that little information is provided about the molecular mechanisms involved in the observed effects, and the detection of drugs with new mechanisms always required new models.

It is generally believed that the costs of developing new pharmaceutical drugs are exploding, while the output of new drugs is actually decreasing. A change in paradigm, the *target-based or mechanism-based drug discovery approach*, was therefore welcomed with great enthusiasm. The techniques of combinatorial chemistry could generate thousands of compounds to be tested against thousands of targets using high-throughput and ultra-high-throughput technology with tremendous capacity. This made it highly effective for the identification of target-selective compounds. However, despite the fact that this approach is very advantageous from a scientific and practical viewpoint, it did not translate into a high success rate in the discovery of new drugs. This has naturally led to questions regarding the success of target-based drug discovery and, more importantly, a search for alternatives.

The target-based approach has therefore been replaced again by the *physiology-based approach*, the classical drug discovery paradigm, or the *function-based approach*, which seeks to induce a therapeutic effect by normalizing a disease-specific abnormality.

On top of this, important changes in the management of some of the larger drug companies have taken place in recent years. Modern managers were installed as chief executive officers and other high-level executives, quite often with little or no biological and technical experience. They were more interested in blockbusters

and in shareholders' value than finding new drugs, especially for rare diseases. Research workers were confronted with cumbersome and inflexible organizational structures characterized by regimentation, control, conformity, and excessive bureaucracy. Consequently, creativity and productivity decreased in this environment. Recently, however, there are signs that the situation is changing.

In this book we ask the question: "Quo vadis, pharmacology?" We suggest giving pharmacologists the freedom, time, and money to carry out their own ideas. We also describe animal models of rare diseases that enable the medically trained pharmacologist to find appropriate drugs.

We are well aware that the rapid progress in biology will once again change the methodological approach in the coming years, and that electronic media will continuously help the researcher to access and share information. However, it is becoming more and more evident that many young pharmacologists have only limited training in classical pharmacological methodologies. When searching for these methods, researchers will only find insufficient information on the methodological details in the electronic databases currently available. To this end, we hope the current book may bridge this gap by comprehensively covering those pharmacological methods utilized for over more than a hundred years.

At this point I would like to express my sincere thanks to all colleagues who contributed to the new and to the earlier editions of this book. Their names and affiliations are given in alphabetical order.

Spring 2007

H. Gerhard Vogel

Preface to the Second Edition

The first edition of “Drug Discovery and Evaluation – Pharmacological Assays” has been well accepted by a broad readership ranging from experienced pharmacologists to students of pharmacology. Therefore, already after a short period of time the question for a second edition arose.

The first edition was mainly centered around the personal laboratory experience of the editors and the contributors. The second edition tries to close evident gaps. The input of biochemistry to pharmacology has grown. Molecular pharmacology puts more emphasis on the mode of action of drugs, albeit it becomes clear that the activities of most drugs are not confined to one single mode of action. Studies in single cells become more and more popular, however, they do not cover the complexity of a whole organism. Possible side effects of drugs can be better detected in whole animals than in single cells. Therefore, the new requirements of the health authorities on safety pharmacology put emphasis on experiments not only in whole anesthetized animals but in conscious ones. The second edition of this book takes note of these requirements and devotes special chapters for each indication to safety pharmacology.

Molecular biology also introduced new methods to pharmacology, such as the polymerase chain reaction (PCR), reverse PCR, Northern, Western and Southern blotting. Very recently, microarray technology, proteomics, and mass spectroscopy were added as novel *in vitro* methods. Furthermore, genetically modified animals have been created which resemble human diseases. Pharmacogenomics has already begun to influence pharmacology and even will have greater input in the future. Special attention is given to these new achievements in various chapters of the book.

The editor and the co-editors are well aware that the rapid progress in biology during the next decades will change the methodological approach. Electronic media will help the researcher for continuous information. However, it becomes more and more evident, that young pharmacologists have only insufficient training in the classical pharmacological methodology. Searching for these methods, e.g., for safety pharmacology, the researchers cannot find sufficient information on the methodological details in the electronic data bases currently available. This book covering the pharmacological methods of more than hundred years may be of help.

The guidelines concerning the care and use of laboratory animals have been updated.

A change in paradigm of pharmacological research has been claimed but superiorities of these new approaches compared to the old ones have still to be proven. To address this, an introductory chapter on new strategies in drug discovery and evaluation has been added, including combinatorial chemistry; high throughput screening, ultrahigh throughput screening and high content screening; pharmacogenomics, proteomics and array technology. Some critical thoughts on errors in screening procedures have been added.

At this place, we would like to express our sincere thanks to all colleagues who contributed to the new and to the first edition of this book. Their names and their positions are given alphabetically.

March 2002

H. Gerhard Vogel
Also in the name of the co-editors

Preface to the First Edition

This book is intended to be an aid for experienced pharmacologists as well as for newcomers in the field of experimental pharmacology. The student in pharmacology, the pharmacist and the medicinal chemist will find a survey of pharmacological assays that can be used for a given indication and for which methods have demonstrated their relevance. The researchers working in special fields of pharmacology will find assays in other, unfamiliar areas which might help to expand their own research.

Certain therapeutic domains, such as cardiovascular, respiratory and renal disorders, psychiatry and neurology, peripheral nerve function, pain and rheumatic diseases, metabolic and endocrine diseases including diseases of the gastrointestinal tract, are discussed in this book.

Each chapter is divided into pharmacological classes, e.g., anxiolytics, anti-epileptics, neuroleptics, antidepressants, or anti-Parkinson drugs. For each class, *in vitro* methods, tests on isolated organs and *in vivo* methods are described.

For each method the purpose and rationale are given first, followed by a description of the procedure, evaluation of the data, modifications of the method described in the literature, and the relevant references. If possible, a critical assessment of the method based on personal experience is added. The hints for modifications of the method and the extended reference list will be of value for the experienced pharmacologist.

A few words for the justification for a book of this kind: In 1959, A.J. Lehman, Director of the Division of Pharmacology at the Food and Drug Administration, USA, wrote:

... Pharmacologists are individualists. Like most scientists they are seldom willing to copy each other's techniques in detail, and so their methods vary from one to the other. Nevertheless, there are basic principles and techniques which must be applied to establish the safety of a new drug.

Visitors could also read a sticker in his office:

You too can learn pharmacology, in only three lessons: each of them lasting ten years.

Pharmacologists have always used methods from neighboring disciplines; in the past, e.g. from anatomy, pathology, surgery, zoology and predominantly physiology. Useful methods also came from electrophysiology and the behavioral sciences. Earlier drug discovery was almost exclusively based on animal experiments, clinical observations and serendipity.

In recent years, a major input has come from biochemistry. The effect of many drugs in human therapy could be explained biochemically as effects on specific enzymes or receptors. With the detection of more and more receptor subtypes, the

activity spectrum of a single compound became more and more complicated. At present, molecular biology provides pharmacologists with human receptors and ion channels expressed in mammalian cells in culture. This avoids the apparently existing species differences, but the multitude of natural and perhaps artificial subtypes raises the question of physiological and pathological relevance.

The challenge for the pharmacologist always will be to correlate *in vitro* data with *in vivo* findings, bearing in mind the old saying: "*In vitro simplicitas, in vivo veritas*". The effects found in tissue cultures are quite often not typical for an intact organism.

Pharmacologists, especially in industry, have the task to find new drugs for human therapy by using appropriate models. Pharmacological models have to be relevant, that means they should predict the intended therapeutic indications. A pharmacological model can be considered relevant or correlational, if the effects obtained correlate with results observed in human therapy.

To be relevant or "correlational", a model has to fulfill some basic criteria:

- First, the model must be sensitive in a dose-dependent fashion to standard compounds that are known to possess the desired therapeutic property.
- Second, the relative potency of known active agents in the model should be comparable to their relative potency in clinical use.
- Third, the model should be selective, i.e. the effects of known agents in this therapeutic indication should be distinguishable from effects of drugs for other indications. Positive data with a new compound allow the prediction of a therapeutic effect in patients.

If new assays are applied to indications for which no effective drug is known, there must be sufficient evidence that this model is relevant for the pathological status in this indication.

The methods presented in this book have been selected according to these criteria.

Considerable discussion is going on about the necessity of animal experiments. One has to accept that only the whole animal can reflect the complexity of a human being. Even an experiment with human volunteers is only a model, albeit a highly relevant one, to investigate therapeutic effects in patients. The degree of relevance increases from isolated molecules (e.g. receptors or enzymes) to organelles, to organs up to conscious animals and human volunteers.

Without any doubt, animal experiments are necessary for the discovery and evaluation of drugs. However, they should be performed only if they are necessary and well conceived.

In Chapter N, regulations existing in various countries concerning the care and use of laboratory animals are listed. Furthermore, guidelines for anesthesia, blood collection and euthanasia in laboratory animals are given. In carrying out animal experiments, one must adhere strictly to these guidelines. Following these rules and planning the experiments well, will eliminate or minimize pain and discomfort to the animal. The methods described in this book had the welfare of the animals as well as the benefit of the procedure for the well-being of mankind in mind.

Here, we would like to express our sincere thanks to all colleagues who contributed to this book. Their names and positions are given alphabetically below.

Autumn 1996

*H. Gerhard Vogel,
Wolfgang H. Vogel*

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Contributors

Editor

Prof. emer. Dr. Dr. H. Gerhard Vogel,
Johann Wolfgang Goethe Universität,
Frankfurt,
Germany

Contributors to the third edition

Albus, Udo,
Sanofi-Aventis Pharma Research,
65926 Frankfurt,
Germany

Bickel, Martin,
Sanofi-Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Gögelein, Heinz,
Sanofi-Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Hart, Susan G. Emeigh,
Genaera Corporation,
5110 Campus Drive Plymouth Meeting,
PA 19462,
USA

Herling, Andreas W.,
Sanofi-Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Huwiler, Andrea,
Institute of Pharmacology University of Bern,
Friedbühlstrasse 49,
3010 Bern,
Switzerland

Juretschke, Paul,
Sanofi-Aventis Pharma Research,
65926 Frankfurt,
Germany

Mousa, Shaker A.,
The Pharmaceutical Research Institute at Albany,
Albany College of Pharmacy,
106 New Scotland Avenue,
Albany,
NY 12208,
USA

Müller, Günter,
Sanofi-Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Sandow, Jürgen,
Sanofi-Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Schäfer, Hans-Ludwig,
Sanofi-Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Schultz, Beth,
College of Optometry Philadelphia,
60 Tennis Ave Ambler,
PA 19002,
USA

Vogel, Wolfgang F.,
Department of Laboratory Medicine and Pathobiology,
Faculty of Medicine,
University of Toronto,
Canada

Vogel, Wolfgang H.,
Jefferson Medical College,
Thomas Jefferson University,
Department of Pharmacology,
Philadelphia,
USA

Wirth, Klaus J.,
Sanofi-Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Contributors to the second edition

Albus, Udo,
Aventis Pharma Research,
65926 Frankfurt,
Germany

Bickel, Martin,
Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Bleich, Markus,
Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Dere, Ekrem,
Institute of Physiological Psychology,
Heinrich Heine Universität Düsseldorf,
Germany

Gögelein, Heinz,
Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Herling, Andreas W.,
Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Hropot, Max,
Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Laux, Volker,
Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Just, Melitta,
Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Müller, Günter,
Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Rieß, Günther,
Aventis Pharma Research,
65926 Frankfurt,
Germany

Sadow, Jürgen,
Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Schäfer, Hans-Ludwig,
Aventis Pharma Research,
Metabolism Research,
65926 Frankfurt,
Germany

Vogel, Wolfgang F.,
Assistant Professor,
Department of Laboratory Medicine and Pathobiology,
Faculty of Medicine,
University of Toronto,
Canada

Vogel, Wolfgang H.,
Jefferson Medical College,
Thomas Jefferson University,
Department of Pharmacology,
Philadelphia,
USA

Wirth, Klaus J.,
Aventis Pharma Research,
Cardiovascular Research,
65926 Frankfurt,
Germany

Contributors to the first edition**Albus, Udo,**

Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Bartlett, Robert R.,

Hoechst-Marion-Roussel,
Immunopharmacology,
65174 Wiesbaden,
Germany

Bickel, Martin,

Hoechst-Marion-Roussel,
Metabolism Research,
65926 Frankfurt,
Germany

Brioni, Jorge D.,

Abbott Laboratories,
Neuroscience Research,
Abbott Park,
IL 60064,
USA

Gögelein, Heinz,

Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Greger, R.,

Institute of Physiology,
University of Freiburg,
79104 Freiburg,
Germany

Herling, Andreas W.,

Hoechst-Marion-Roussel,
Metabolism Research,
65926 Frankfurt,
Germany

Hock, Franz Jakob,

Hoechst-Marion-Roussel,
General Pharmacology,
65926 Frankfurt,
Germany

Hropot, Max,

Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Huger, Francis P.,

Hoechst-Marion-Roussel,
Neuroscience Research,
Sommerville,
NJ,
USA

Jablonka, Bernd,

Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Just, Melitta,

Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Linz, Wolfgang,

Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Maas, Jochen,

Hoechst-Marion-Roussel,
Preclinical Development,
65926 Frankfurt,
Germany

McGaugh, James L.,

University of California at Irvine,
Irvine,
CA 92717,
USA

Müller, Günter,

Hoechst-Marion-Roussel,
Metabolism Research,
65926 Frankfurt,
Germany

Raiss, Ruth X.,

Hoechst-Marion-Roussel,
Osteoarthritis Research,
65174 Wiesbaden,
Germany

v. Rechenberg, Wolfrad,
Hoechst-Marion-Roussel,
Metabolism Research,
65926 Frankfurt,
Germany

Rudolphi, Karl A.,
Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Schölkens, Bernward A.,
Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Seeger, Karl,
Hoechst-Marion-Roussel,
Pharma Research,
65926 Frankfurt,
Germany

Weithmann, K.U.,
Hoechst-Marion-Roussel,
Immunopharmacology,
65174 Wiesbaden,
Germany

Wirth, Klaus J.,
Hoechst-Marion-Roussel,
Cardiovascular Research,
65926 Frankfurt,
Germany

Introduction

Strategies in Drug Discovery and Evaluation¹

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0.1 Historical Approaches in Drug Discovery

Today's medicine is based on traditional medicine. Traditional medicines exist in every continent of the globe and in every cultural area of the world. The most famous ones are traditional Chinese medicine in East Asia, Ayurvedic medicine in India, and formerly Galenic medicine in Europe, all of which have some resemblance to one other (Vogel 1991).

Each of these traditional medicines has its own origins and an individual basic philosophy. The art of practicing **Chinese medicine** stretches back over several thousand years. The legendary culture hero, Shen-nong, is said to have tested many herbs for their medical properties. *Pen-ts'ao*, the first compilation of herbal medicines, is connected with his name (Unschuld 1973, 1986). Since Ancient times, the Chinese have divided the world into five symbolic elements: Wood, Fire, Earth, Metal and Water. Everything in the world is dominated by one of these elements, and their constant interplay, combined with those of yin and yang, explain all change and activity in nature. The positive, generative cycle proceeds as follows: Wood burns to generate Fire; Fire produces ashes which generate Earth; Earth generates Metal, which can be mined from the ground; when heated Metal becomes molten like Water; Water promotes the growth of plants, thereby generating Wood. The negative subjugable cycle is complementary to the positive, generative one. Chinese medical views regarding the vital internal organs are based on the theories of *yin* and *yang*, the five elements (which are each related to body organs and colors), and the meridians. With medicines consisting mainly of herbal drugs and minerals, Chinese doctors manipulate these natural relationships to adjust energy imbalances caused by the excess or deficiency of these forces in the body. Chinese physicians and philosophers developed a special system of physiology describing vital organs as storage houses and vital connections as meridians that became the basis of acupuncture (Porkert 1973).

In India *Ayurveda*, *Siddha* and *Unani* systems of medicine provide healthcare for a large part of the population. The word **Ayurveda** is composed of two parts: Ayu (= life) and Veda (= knowledge). Scholars of Ayurveda had placed the origins of this science of life at some time around 6000 BC. They were orally transmitted by successive generations. The principles were recorded in great detail in compendia, which are called *Samhitas* (Dash and Junius 1987; Da-

¹With contributions by P. Juretschke, W.F. Vogel, and G. Riess.

hanukar and Thatte 1989; Mazars 1994). Compared with modern anatomy and physiology Ayurveda is based on certain fundamental doctrines, known as the *Darshanas*, such as the seven *Dhatus*. They can be described not exactly as organs but as body constituents. The three *Doshas*: *vata*, *pitta* and *kapha* are regulators of cell function in various ways. A balance of the three doshas is essential for maintaining health. Imbalance of the doshas creates disease. Drug therapy in Ayurveda is highly individualized. The choice of drugs as well as the dose is not only influenced by the disease process, but also by the constitution of individuals and environmental conditions which affect the balance of the doshas and therefore the response to drugs.

Both traditional Chinese and Ayurvedic medicine developed further in terms of formulations. There is also the tendency to adopt the modern forms of clinical trials, but there has never been a change in paradigm as far as the basic philosophy is concerned.

Traditional European medicine goes back to the time of Egyptian and Babylonian-Assyrian culture. About 3000 BC the Sumerians developed a system of cuneiform writing that enabled them to write on clay tablets. At the time of King Assurbanipal 2000 BC a first comprehensive *Materia Medica* was written containing approximately 250 vegetable drugs and 120 mineral drugs (Koecher 1963). Around 1500 BC, the famous Egyptian papyrus *Ebers* was written, describing more than 700 drugs drawn from plants, animals, and minerals. Some are still used in our time, such as garlic and poppy seeds.

Greek philosophers such as Empedocles, Aristoteles and Pythagoras, all from around 500 BC, influenced European medicine a great deal. They created the theory of the Four Elements, which were proposed to be the components of all matter, including animals and Man. These Four Elements were Water, Air, Fire and Earth (Schöner 1964). The most famous physicians were Hippocrates (around 400 BC) and Galenus, living in the second century after Christ. In medieval times the system of pathology and therapy, originating from Galenus, dominated Western medicine. The fundamental principle in so-called Galenism was the transformation of humoral pathology into a rigid dogma (Siegel 1968). The school of Hippocrates had already formulated the theory of the Four Humours (paralleling the Four Elements), the correct balance of which meant health, while every disturbance of this balance caused disease. There were Four Humours: Blood (coming from the heart), Phlegm (supposed to come from the brain), Yellow Bile (supposed to be

secreted from the liver), and Black Bile (supposed to come from the spleen and the stomach). Each of these humours had definite qualities. Blood was moist and warm; Phlegm, moist and cold; Yellow Bile, warm and dry; Black Bile, cold and dry. Furthermore, there was a definite connection between the predominance of one humour in the metabolic system and an individual's temperament, such as sanguine, phlegmatic, choleric, and melancholic.

European medicine has been influenced by Arabian medicine, mainly by the physician Ali Ibn Sina, who was known in Europe under the name Avicenna. His books were translated into Latin and his *Canon Medicinae* has influenced European medicine for centuries (Gruner 1930).

Saint Hildegard of Bingen, the abbess of a monastery, was one of the most famous physicians and pharmacists in the twelfth century (Müller 1982). She wrote many books on human nature and the use of herbal drugs. Theophrastus Bombastus von Hohenheim, also called Paracelsus and who lived in the fifteenth century, was a reformer of European medicine (Temkin 1941). One of his ideas was that in nature a remedy could always be found against every disease. The Latin sentence "*Ubi malum, ibi remedium*" was the core idea of the signature theory that the shape or the color of a plant indicates against which disease the herbal drug can be used.

European medicine has undergone a great change in paradigm. In contrast to traditional Chinese medicine and Ayurveda, traditional European medicine no longer exists and is only a matter of history of medicine. Even the self-understanding of modern phytotherapy in Europe is completely based on the allopathy that means modern Western medicine.

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Classical Pharmacological Approaches

The most important achievements of **modern Western medicine** were made in several areas such as diagnosis, infectious diseases, endocrinology and medicinal chemistry. Virchow founded cellular pathology in the nineteenth century (Pagel 1906). The intensive use of the microscope in medicine with histological comparison of diseased and normal organs allowed the change from humoral to **cellular pathology**.

Medicinal chemistry as an important science started less than 100 years ago. The active principles of plants, mostly alkaloids, were isolated and were the starting point for syntheses, such as:

morphine and papaverine from *Papaver somniferum* for synthetic analgesics and spasmolytics; atropine from *Atropa belladonna* for synthetic spasmolytics; cocaine from *Erythroxylon coca* for synthetic local anesthetics; quinine and quinidine from *Cinchona succirubra* for synthetic anti-malaria drugs and antiarrhythmics; ephedrine from *Ephedra sinica* for synthetic sympathomimetics and sympatholytics including β -blockers, xanthines such as caffeine; theobromine and theophylline from *Coffea arabica*, *Theobroma cacao*, *Camellia sinensis* for vasotherapeutics such as pentoxifylline; ergot alkaloids from *Claviceps purpurea* for semi-synthetic ergot derivatives; reserpine and ajmaline from *Rauwolfia serpentina* for synthetic antihypertensives and antiarrhythmics; physostigmine from *Physostigma venenosum* for potential antidementia drugs; glycosides from *Digitalis lanata* and *Digitalis purpurea* for semi-synthetic cardiac glycosides; anthraquinones from *Senna angustifolia* or *Rhamnus frangula* or *Rheum officinale* for synthetic laxatives.

Pharmacological research started in Europe in the second half of the nineteenth century when its founders, Rudolf Buchheim and Oswald Schmiede-

berg, investigated the action of existing drugs in animal experiments (Kochwieser and Schechter 1978).

With the emergence of synthetic chemistry the pharmacological evaluation of these products for therapeutic indications became necessary. Many new drugs were discovered by this classical approach during the twentieth century.

The **classical way of pharmacological screening** involves sequential testing of new chemical entities or extracts from biological material in **isolated organs** followed by **tests in whole animals**, mostly rats and mice but also higher animals if indicated. Most drugs in use nowadays in therapy have been found and evaluated with these methods.

The chemistry and pharmacology of drugs found and used in therapy until 1970 are described in the five volumes of the book by Ehrhart and Ruschig H (eds), *Arzneimittel. Entwicklung, Wirkung Darstellung* (1972).

In the mid 1970s **receptor binding assays** were introduced as an approach for compound evaluation by the development of radioligand binding assays, based on evaluation procedures and mathematical calculations provided by Schild (1947), Scatchard (1949), Stephenson (1956), Ariëns and van Rossum (1957), Arunlakshana and Schild (1959), Furchgott (1966), Cheng and Prusoff (1973), Rodbard and Frazier (1975), Lefkowitz and Williams (1977), Bennett (1978), Creese (1978), Munson and Rodbard (1980), McPherson (1985a,b), Tallarida and Murray (1987), and Greenstein (1991). Receptor binding assays were described for various transmitters as well as assays for ion channels and neurotransmitter reporters (e. g., by Snyder et al. 1975; Bylund and Snyder 1976; Enna 1978; U'Prichard et al. 1978, 1979; Bruns et al. 1980; Starke 1987; Dohlman et al. 1991; Krogsgaard-Larsen et al. 1991; Snider et al. 1991; Betz 1992; Wisden and Seeburg 1992; Amara and Arriza 1993; Bowery 1993; Wess 1993; Isom et al. 1994; Köhr et al. 1994; Goto and Yamada 1998; Chittajallu et al. 1999; Bormann 2000).

Characterization and classification of receptors is a continuous procedure (William 1991; Kebedian and Neumeyer 1994; Angeli and Guilini 1996; Trist et al. 1997; Godfraind and Vanhoutte 1998; Watling 1998; Alexander et al. 2001).

The use of radioligand-binding assays has facilitated the design of new chemical entities, especially as the information obtained has been used in deriving molecular models for the structure–activity relationship. The receptor technology provides a rapid means to evaluate small amounts of compound (5–10 mg) di-

rectly for their ability to interact with a receptor or enzyme, independent of its efficacy. But as new assays are developed, it also provides a means to profile the activity of compounds against a battery of binding sites, thereby yielding an *in vitro* radioligand binding profile (Shaw 1992). Receptors have been divided into an increasing number of subclasses (Vanhoutte et al. 1996) so that full characterization of a new chemical entity by receptor pharmacology also needs time and material.

DNA sequencing technology has identified many receptors, mostly belonging to the G-protein-coupled receptor superfamily. Reverse molecular pharmacology and functional genomic strategies are recommended to identify the activating ligands for these receptors (Wilson et al. 1998). The reverse molecular pharmacological methodology includes cloning and expression of G-protein-coupled receptors in mammalian cells and screening these cells for a functional response to cognate or surrogate agonists present in biological extract preparations, peptide libraries, and complex compound preparations.

The ligand-binding assay is a powerful tool in the search for agonists and antagonists for novel receptors, and for identification of novel classes of agonists and antagonists of known receptors. However, it does not differentiate between agonist and antagonist. Ligand-binding mass screening can be adapted for very high throughput. However, well-defined criteria have to be fulfilled to avoid blind alleys (Burch 1991).

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Quo Vadis, Pharmacology?

It is universally bemoaned that development costs for new pharmaceutical drugs are exploding, whilst the output of new drugs is decreasing (Horrobin 2001, 2002, 2003; Warr 2003; Sams-Dodd 2005, 2006; Rish-ton 2005; Schmid and Smith 2005; Lundkvist et al. 2006).

Sams-Dodd (2006) analyzed the strength and limitations of various drug discovery approaches. The *target-based drug discovery approach*, once welcomed as a change in paradigm, has for the past 10–15 years been the dominating approach in drug discovery. However, in the past few years the commercial value of novel targets in licensing deals has fallen dramatically, reflecting the fact that the probability of reaching a clinical drug candidate for a novel target is very low. This has naturally led to questions regarding the success of target-based drug discovery and, more importantly, a search for alternatives.

The *mechanism-based approach*, which corresponds to the target-based approach, screens for compounds with a specific mode of action. The focus on a single mechanism that permits the use of high-throughput screening (HTS) and ultra-HTS technology for screening makes it highly effective for the identification of target-selective compounds. However, despite the fact that this approach is highly advantageous from a scientific and practical viewpoint, it does not translate into a high success rate for novel targets. A success rate of 3% for reaching preclinical development is not attractive. Each drug discovery project usually lasts 2–4 years, and if 33 targets on average have to be evaluated to identify one that can proceed into preclinical development, it means that at least 66 years of research is needed to produce one successful drug discovery project for a novel target. Considering that the attrition rate caused by toxicological effects and lack of clinical effects are also substantial, it means that novel unvalidated targets are not attractive for the pharmaceutical industry.

The *physiology-based approach* was the first drug discovery paradigm, and this has resulted in many effective treatments. It seeks to induce a therapeutic effect by reducing disease-specific symptoms or physiological changes and screens compounds for these properties in animal models that mimic specific aspects of disease symptomatology. The screening is conducted in isolated organ systems or in whole animals. The physiology-based approach suffers from low screening capacity and difficulty in identifying the mode of action of drugs.

The *function-based approach* seeks to induce a therapeutic effect by normalizing a disease-specific abnormality. Compared with the mechanism-based approach, functional parameters represent a higher level of organism complexity because function requires the integrated action of many mechanisms. However, unlike the physiology-based approach, the parameters cannot be compared to the symptoms observed in pa-

tients. The screening capacity cannot be used for library screening.

In **summary**, no clear advantage of one drug discovery paradigm over the others could be demonstrated, because all of them have their specific strengths and weaknesses. The classical approaches cannot be abandoned.

Cuatrecasas (2006) analyzed the reasons for insufficiency of drug discovery and development. The low productivity is certainly not related to available budgets, which have increased 30-fold since 1970. The Food and Drug Administration (FDA) or regulatory issues are not fundamental barriers and contribute only marginally to the decline in drug productivity. Likewise, one cannot blame the current state of scientific advances, which in the last 20 years have been revolutionary.

Much more important are **changes in the approach to management**. Foremost among the issues that cripple drug research and development (R&D) is that while utmost creativity and innovation are required, the R&D is conducted in traditional for-profit corporations that are virtually indistinguishable operationally from those that conduct little or no R&D. Most corporations' top management does not understand the complexities of science, its mode of conduct or objectives, and runs the companies in ways that stifle creativity and innovation.

0.3.1 Managers, Not Leaders

In the 1970s things began to change. Modern managers entered as chief executive officers (CEOs) and other high-level executives, mostly with little or no technical experience. Freedom, spontaneity, flexibility, nimbleness, tolerance, compassion, humor, and diversity were replaced by bulky and inflexible organizational structures characterized by regimentation, control, conformity, and excessive bureaucracy. There are still companies that try to focus on excellent science and that attract first-rate scientists. These are, however, exceptional situations.

Drug R&D thrives in a creative, flexible, and nonautocratic environment. Success depends on individual freedom and inspiration rather than dogmatic leadership.

0.3.2 Pressures From Shareholders

The ownership of public companies consists mainly of shareholders who expect rapid (and substantive) re-

turns on their investments. This contrasts (and often conflicts) with the nature of the business objectives, which must be based on long-term investments in science and technology.

0.3.3 Merger Mania

The decreasing earnings have stimulated mergers and acquisitions, driven by the desire to acquire existing sales (products) while decreasing costs via layoffs. This has created conditions that catalyze further inefficiencies and suffocation of innovation. The merged megacompanies' research organizations must be integrated rapidly and redundancies eliminated, often times in haste.

0.3.4 Blockbuster Mania

Companies have become much less interested in developing drugs that will sell less than \$1 billion a year. Without these "blockbusters" they cannot maintain the traditionally high profits. Berkowitz and Sachs (2002) described discovery and development of the blockbuster drug omeprazole.

0.3.5 Orphan Drugs and Drug Pricing

In the 1960s through the 1990s, orphan diseases were simply rare diseases for which drug companies did not wish to initiate discovery programs, for commercial reasons. It was understood that prices could not be adjusted out of line with the prevailing standards. In 1983, the US Congress passed The Orphan Drug Act to provide tax, funding, and exclusivity incentives to companies developing drugs for low-prevalence diseases (defined as fewer than 200,000 cases) that were not expected to provide investment returns. However, many drugs that have become blockbusters had early histories of major disinterest and skepticism.

There are many publications on orphan drugs and drugs for rare diseases (Von Oehsen 1989; Weinstein 1991; Haffner 2002; Rados 2003; Thoene 2004; Fischer et al. 2005; Gericke et al. 2005; Hughes et al. 2005; Moran 2005; Sheehan 2005; McCabe et al. 2006; Reidenberg 2006; Stolk et al. 2006), but they concern mainly cost-effectiveness and funding of clinical trials, but not pharmacological methods. It seems necessary that experimental pharmacologists know rare diseases and think about methods to evaluate drugs against rare diseases.

Researchers are now looking back to the **golden age of drug discovery in the 1950s and 1960s** of the last century. What was different at that time?

A few personal remarks: in the 1960s, we were looking for analgesics in a special chemical series. However, we were afraid of addictive and hallucinogenic potential. In animal experiments we found a compound almost as effective as methadone. After 2 weeks of toxicity tests, we gave it to one of our behavior cats. The results were unequivocal. So, without informing anybody, one of us took a rather high dose orally. He did not hallucinate. So we gave the compound to patients. There were no side-effects after oral dosing, but hallucinogenic side-effects were reported after intravenous dosing. We had to drop development.

Another example: we were looking for an effective and highly tolerable local anesthetic.

One pharmacologist performed animal experiments, but he injected interesting compounds into his forearm to evaluate pain and the local anesthetic effect. An excellent compound was found this way (R. Muschaweck, pers. comm. 2007).

One further example: one of our colleagues was testing sedative and hypnotic compounds. After some toxicological studies, he gave the compound to a colleague in charge of first clinical trials. He took the compound himself, but then collapsed behind his desk (E. Lindner, pers. comm. 2007).

Conclusion: we were not afraid to take our compounds ourselves even flaunting regulations. Thus **compounds were tested by the pharmacologists themselves**.

If we look back to the great breakthroughs in the 1950s and 1960s, we see that many important ones came from **serendipitous** findings.

Horace Walpole (1717–1797) coined the term serendipity in 1754 in allusion to an ancient oriental legend of the “Three Princes of Serendip”. “Serendipity” in drug discovery implies the finding of one thing while looking for something else (Holubar 1991; Ban 2006).

The diuretic action of sulfanilamide and **acetazolamide** as inhibitors of carbonic anhydrase was found in clinical trials (Schwartz 1949; Maren 1960, 1967; Relman et al. 1960).

Sulfonylureas were tested as antibiotic drugs to be compared with sulfonamides. The blood sugar-lowering-effect of **sulfonylureas** designed as antibacterial agents (Loubatières 1946; Franke and Fuchs 1955; Achelis and Hardebeck 1955) was found just by chance. Then it turned out that the amino group was not necessary, resulting in **tolbutamide**. On the other

hand, one has to keep in mind that millions of diabetics were treated successfully with sulfonylureas over decades, whereby only the release of insulin from the pancreatic β -cell, but not the molecular mechanism, was known (Pfeiffer et al. 1959; Bänder et al. 1969).

The antipsychotic activity of phenothiazines (**chlorpromazine**) originally tested as antihistaminic drugs was found in clinical studies (Delay and Deniker 1952; Laborit et al. 1952; Courvoisier 1956), as was the antidepressant effect of **imipramine** (Kuhn 1958).

The antidepressant effect of the monoamine oxidase inhibitor **iproniazid** was found in clinical trials (Kline 1958; Bailey et al. 1959; Dick 1959).

Valproic acid was used as an organic solvent in research laboratories for eight decades, until the observation of action against pentylentetrazol-induced convulsions in rodents. Clinical experience emphasized therapy for absence seizures in primary generalized epilepsies. Anecdotal observations in patients with both epilepsy and migraine headaches led to the discovery of its antimigraine efficacy. More than a decade later, clinical trials established a significant efficacy of valproate in mania (Henry 2003).

Butazolidin was used as an additive to increase the solubility of aminopyrine resulting in a combination product (Wilhelmi 1949, 1950). The therapeutic value of butazolidin as single drug was recognized later (Kuzell and Schafferzick 1952; Belart 1953).

The blood-pressure-lowering effect of **clonidine** was found when tested as a vasoconstrictive drug for nasal application. The mechanism of action was clarified in animal experiments (Kobinger and Walland 1967; Callingham 1971; Timmermans and van Zwieten 1980, 1981; Langer and Hicks 1984).

Furosemide was found to be a potent diuretic and saluretic drug comparable to other substances. But animal experiments and studies in volunteers showed an effect at doses at which other diuretic drugs were no longer effective. Thus the name “**high-ceiling diuretic**” was coined.

The discovery of **sildenafil**, a highly selective inhibitor of phosphodiesterase-5 (PDE-5), was the result of research on chemical agents that might be useful in the treatment of coronary heart disease. Initial clinical studies on sildenafil were not promising with respect to its anti-anginal potential. However, the incidental discovery of its anti-impotence effect led to its approval for the treatment of erectile dysfunction (Campbell 2000; Enna 2000; Raja and Nayak 2004).

Most new drugs, however, were found by the **classical approach**, which has the advantage of relatively high relevance. If a compound has blood-pressure-

lowering activity in hypertensive rats after oral dosage, the chances of activity in humans are high. Measurement of dose–response curves, effects over a given period of time, and comparison of the effects after intravenous and oral administration have already given hints for pharmacokinetic data. This approach had the disadvantage that it was time-consuming and required relatively large amounts of the new compound (usually about 5 g). Furthermore, this approach provided little information about the molecular mechanisms involved in the observed effects.

What were the **deficiencies of the classical approach**?

We did not know the exact mode of action of our drugs in spite of the fact that we could treat patients effectively.

This **situation has changed**.

Molecular biology has a significant impact on drug discovery, offering us over 30,000 genes and their protein products as potential drug targets, and revolutionizing the processes involved in seeking drug targets, testing hypotheses, and screening for activity. In addition, the techniques of combinatorial chemistry are available, which generate thousands of compounds to test against the thousands of targets. The search for new lead compounds involves intricate high-throughput screening technology with tremendous capacity, and the use of cell culture and recombinant human cells and enzymes (In Vivo Pharmacology Training Group 2002). So we have many hypotheses and we know many biochemical steps that could be the mode of action of a new drug, but we do not know the new drug. The biochemical approach reduced the use of animals. When animal experiments are performed at a later stage, quite often the results of *in vitro* experiments cannot be validated *in vivo*.

In all countries we have regulations for the care and use of laboratory animals (see Chap. Q of this book). Animal rights extremists have created an atmosphere where students are reluctant to perform whole-animal studies. Our experience tells us that only a research worker who loves animals and is not afraid of them should perform animal experiments.

What can be **recommended** from experimental pharmacology to **increase the efficacy of drug development**?

- Looking for **short-cuts**, even neglecting the present rules when performing self experiments.
- To give pharmacologists the freedom, time and money to **realize their own ideas**.
- Giving room for **serendipity**.

- Performing **animal experiments** in parallel to *in vitro* studies.
- In respect to **orphan drugs**, it seems necessary that experimental pharmacologists know rare diseases and think about methods to evaluate drugs against these diseases.
- Pharmacologists should have **training in medicine**, not only in biochemistry.
- Outstanding pharmacologists should be selected as **leaders in research and development**.

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0.4

New Approaches in Drug Discovery

0.4.1

Combinatorial Chemistry

It was always due to the ingenuity of the chemist that new chemical entities in pharmaceutical research were created (Schapira et al. 2000; Schreiber 2000). For some period of time, computer-based molecular modeling was thought to be the most effective approach. With the increasing characterization of the three-dimensional structures of receptors and enzymes, the design of molecules that interact with these biological targets was thought to be an intellectual approach using modern computer technology (Stigers et al. 1999).

This attitude to the development of **combinatorial chemistry** has changed. It has been defined as the systematic and repetitive covalent connection of a set of different “building blocks” of varying structures to each other to yield a large array of diverse molecular entities (Gallop et al. 1994; Gordon et al. 1994; Fecik et al. 1998).

An interim analysis (Adang and Hermkens 2001) showed only limited success of combinatorial chemistry approaches in terms of delivering leads. The interest in natural products has been regained (Ortholand and Ganesan 2004; Piggott and Karuso 2004).

It has become possible to use solid- or solution-phase syntheses with different chemistries and scaffolds to produce libraries tailor-made for finding or optimizing a lead directed at almost any class of target (Hogan 1996; Maclean et al. 1997; Appleton 1999; Dooley and Houghten 1999; Lukas et al. 1999; Beeley and Berger 2000; Houghten 2000; Lazo and Wipf 2000; Rademann and Jung 2000).

Several suggestions were made to enhance the success of the hit to lead process (Goodnow 2001; Goodnow et al. 2003; Alanine et al. 2003; Bleicher et al. 2003). Golebiowski et al. (2003) reviewed lead compounds discovered from libraries at various research institutes. Design of screening libraries targeted at macromolecules, such as synthetic and G-protein-coupled receptors, was described by Juliano et al. (2001), Crossley (2004), Jimonet and Jäger (2004), and Srinivasan and Kilburn (2004). Purification strategies for combinatorial and parallel chemistry were discussed by Edwards (2003).

New tools, such as molecular docking algorithms (Burkhard et al. 1999), mapping of protein-binding sites by nuclear magnetic resonance (Shuker et al. 1996), preparation of highly enantioselective selectors for chiral HPLC (Lewandowski et al. 1999), microfluidic systems (Watts and Haswell 2003; Cullen et al. 2004), light-directed synthesis (LeProust et al. 2000), and homology modeling of proteins (Kiyama et al. 1999), allow an unprecedented level of rational design to guide the synthesis of prospective drugs.

Spectroscopic methods, including especially mass spectrometry (Mathur et al. 2003; Schefzick et al. 2004) and to a lesser extent infrared and nuclear magnetic resonance, have been applied at different levels of combinatorial library synthesis: in the rehearsal phase to optimize the chemistry prior to library generation, to confirm library composition, and to characterize after screening each structure that exhibits positive response (Dancík et al. 1999; Hajduk et al. 1999; Loo et al. 1999; Scherer et al. 1999; Cancilla et al. 2000; Enjalbal et al. 2000; Kyranos et al. 2001). Schmid et al. (2000), Heeren et al. (2004), and Zhang et al. (2005) discussed the application of mass spectrometry using high-performance Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) methods.

Blundell and Patel (2004) underlined the value of high-throughput X-ray crystallography for drug discovery.

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0.4.2 High-Throughput Screening, Ultra-High-Throughput Screening, and High-Content Screening

The logical development of receptor technology was high-throughput screening (HTS) (Bolger 1999; Broach, Thorner 1996; Cox et al. 2000; Major 1999). This evolution was closely connected with the changes in strategy of chemical synthesis. The vast number of compounds produced by combinatorial chemistry and the possibility of testing many compounds, including natural products (Harvey 2000), in a short period of time by HTS stimulated each other.

High-throughput screening is the process of testing a large number of diverse chemical structures against disease targets to identify “hits”. Compared to traditional drug screening methods, HTS is characterized by its simplicity, rapidness, taking the ligand–target interactions as the principle, as well as leading to a relatively high information harvest. As a multidisciplinary field, HTS involves an automated operation platform, a highly sensitive testing system, specific screening models (*in vitro*), an abundant components library, and a data acquisition and processing system. Various technologies, especially technologies such as fluorescence, nuclear magnetic resonance, affinity chromatography, surface plasmon resonance, and DNA microarray, are available, and the screening of more than 100,000 samples per day is possible. Fluorescence-based assays include the scintillation proximity assay, time-resolved energy transfer, fluorescence anisotropy, fluorescence correlation spectroscopy, and fluorescence fluctuation spectroscopy.

With the introduction of robotics, automation and miniaturization techniques, it became feasible to screen 50,000 compounds a day with complex workstations. Full automation of all assay steps, from compound addition to data collection, ultimately allows screens to be run continuously – 24 h a day, 7 days a week. Another progress is the development of 1536-well microtiter plate formats (Berg et al. 2000; Dunn

et al. 2000). Multiple types of formats are emerging beside the 96-, 384-, and 1536-, even 3456- and 9600-well plates (Sills 1998).

The trend toward assay miniaturization for high-throughput and ultra-high-throughput screening intensified the development of homogenous, fluorescence-based assays in higher density, smaller volume microplate formats. Microfluidic devices have been designed to perform continuous-flow biochemical and cell-based assays providing orders of magnitude reduction in reagent consumption (Sundberg 2000; Cullen et al. 2004). Harding et al. (1997) described the development of an automated HTS system involving two systems, the first handling isotopic assay (“hot” system), the second non-isotopic assays (“cold” system).

An ultra-high-throughput screen based on fluorescence isotropy and performed entirely using 1536-well assay plates was published by Turconi et al. (2001). Further studies on miniaturized HTS technologies were published by Wolcke and Ullmann (2001) and Brandish et al. (2006).

Target validation should not be underestimated in the drug research and development process (Williams 2003).

Enzyme assays for HTS were evaluated by Goddard and Reymond (2004). Assaying enzyme-catalyzed transformation in high throughput is crucial to enzyme discovery, enzyme engineering and the drug development process. In enzyme assays, catalytic activity is detected using labeled substrates or indirect sensor systems that produce a detectable spectroscopic signal upon reaction.

High-content screening was proposed by Giuliano et al. (1997) as a new approach to easing key bottlenecks in the drug discovery process. High-content screening defines the role of targets in cell function by combining fluorescence-based reagents with the ArrayScan™ to automatically extract temporal and spatial information about target activities within cells. High-content screening integrates cell-based assays, high-resolution fluorescence microscopy, and proprietary image processing algorithms for the automated analysis of cellular and subcellular events. It delivers the potential to screen using targets that previously were minimally used or avoided due to a lack of a robust way to measure them, such as morphology changes, cellular differentiation and cytoskeletal changes, cell-to-cell interactions, chemotaxis and motility, as well as spatial distribution changes such as receptor trafficking or complex formation.

Clark and Pickett (2000) underlined the importance of computational methods for the prediction of “drug-

likeness”, in particular for the prediction of intestinal absorption and blood–brain barrier penetration.

Several authors expressed their thoughts on the future of HTS (Divers 1999; Eglén 1999; Fox et al. 1999, 2001; Stahl 1999; Mander 2000). Valler and Green (2000) expressed some doubts on diversity screening in drug discovery and proposed focused screening using three-dimensional information about the target which may improve the hit rate 10- to 100-fold over random screening.

Nevertheless, this technical progress has led to a new concept in drug discovery: “a change in paradigm”. Large numbers of hypothetical targets are incorporated into *in vitro* or cell-based assays and are exposed to large numbers of compounds of natural or synthetic origin.

This change in paradigm created new areas, such as bioinformatics, cheminformatics, and functional genomics (Ohlstein et al. 2000, 2006; Ryu and Nam 2000; Blake 2004).

Many compounds can elicit a positive response in a particular assay, which is called a hit. In this new terminology “**hit**” is a molecule with confirmed activity from the primary HTS assay, a good profile in secondary assays and a confirmed structure.

The hits have to be structurally defined and may give rise to leads (Lahana 1999). “**Lead**” is a hit series for which the structure–activity relationship is shown and activity demonstrated both *in vitro* and *in vivo*.

The implementation of these methods is being complemented by an increase in the use of automation and robotics. Automation may be defined as the use of stand-alone instrumentation (work stations) that performs a given task, whereas robotics uses a robot arm or track systems to move microplates between these instruments. These technologies are continuously improved (Brown and Proulx 1997; Lightbody and Alderman 2001; Ruch 2001; Alanine et al. 2003).

High-throughput screening methods are also used to characterize metabolic and pharmacokinetic data about new drugs (Watt et al. 2000; White 2000). Kansy et al. (1998) recommended parallel artificial membrane permeation assays for the description of passive absorption processes early in HTS.

High-throughput screening assays have been used for the assessment of cytochrome P450 isoenzyme metabolism using fluorogenic Vivid substrates (Marks et al. 2002, 2003, 2004).

A large pharmaceutical company has the potential to screen up to 100,000,000 compounds per year (Drews 2000). In any case, the leads or derivatives of them have to be tested in more complex mod-

els, isolated organs or animals. Only compounds active in classical pharmacological tests can be taken into preclinical and clinical development. Since only a few drug candidates, originally screened in HTS, have reached the market, the searching for higher productivity of HTS is continuing (Fox et al. 2001, 2004; Liu et al. 2004), especially because development costs for new drugs have increased tremendously (DiMasi et al. 2003). With the completion of the human genome sequencing, bioinformatics will be an essential tool in target discovery, and the *in silico* analysis of gene expression and gene function will be an integral part of it, facilitating selection of the most important targets for a disease under study (Terstappen and Reggiani 2001).

Optimistic papers on the value of virtual screening of virtual libraries were written by Green (2003) as well as by Davies et al. (2006). Some doubts were expressed on the philosophy of research management in the pharmaceutical industry neglecting the influence of creative scientists, failing to understand biological complexity, and overestimating the outcome of the “change in paradigm” (Horrobin 2000, 2001, 2002, 2003). Nevertheless, the race to test more and more compounds in a shorter time in ultra-HTS with more automation is continuing.

0.4.2.1 Errors in HTS

As for every screening procedure, HTS suffers from type 1 and type 2 errors. Type 1 errors are false positives. In HTS, a poor candidate or an artifact gives an anomalously high signal, exceeding an established threshold. Type 2 errors are the false negatives. In HTS, a perfectly good candidate compound is not flagged as a hit, because it gives an anomalously low signal. Moreover, a low degree of relevance of the test may induce a high failure rate of type 2 (see also the discussion at the end of this chapter).

Much more attention is given in the literature to false-positive results in HTS than to type 2 errors (Fowler et al. 2002; Lin et al. 2002; Bader and Hogue 2003; Colinge et al. 2003; Francis and Friedman 2003; Glover et al. 2003; Jenkins et al. 2003; Turek-Etienne et al. 2003; Deng et al. 2004; Diller and Hobbs 2004; Vidalain et al. 2004; Huth et al. 2005). Some of the false positives are promiscuous compounds that act noncompetitively and show little relationship between structure and function. McGovern et al. (2002, 2003) and Seidler et al. (2003) found that one mechanism of inhibitor promiscuity is aggregate formation: individual molecules group together to form particles

30–1000 nm in diameter and these aggregates are the active inhibitory species.

0.4.2.2 Statistical Evaluation of HTS

The ability to identify active compounds (“hits”) from large libraries accurately and rapidly is the ultimate goal of HTS assays, which depends on the suitability and quality of the assay.

The Z-factor and the Z' factor were introduced to characterize the reliability of high-throughput assays (Zhang et al. 1999, 2000b).

The Z-factor is a dimensionless, simple statistical characteristic, which reflects the assay signal dynamic range and the data variation associated with the signal measurements. The Z-factor is defined as a screening window coefficient, which uses the formula

$$Z = 1 - \frac{3\sigma_s + 3\sigma_c}{|\mu_s - \mu_c|}$$

in which μ_s and μ_c denote the means of the population and control (usually background) signals, respectively, and the signal standard deviations are denoted as σ_s and σ_c , respectively. This coefficient takes into account the assay signal's dynamic range, the data variation associated with the sample measurement in the presence of the test library, and the data variation associated with the reference control measurement.

The Z'-factor has been defined as the ratio of the separation band to the dynamic range of the assay, based on the positive control and negative control data of the assay. It takes the formula

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

in which μ_{c-} and μ_{c+} denote the means of the negative control signal and positive control signal, respectively. The standard deviations of the signals are denoted as σ_{c-} and σ_{c+} , respectively. The Z'-factor is a simple, dimensionless, and characteristic parameter for the quality of each assay.

Values of 0.7 or higher are considered to indicate a valuable assay.

CRITICAL ASSESSMENT OF THE METHOD

The Z-factor and the Z'-factor have been used by various authors to characterize the quality of assays (Zhang et al. 2000a; Drake et al. 2002; Lin et al. 2002; Kotarsky et al. 2003; Hamid et al. 2004).

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0.4.3

Technologies for High-Throughput Screening

Fundamental changes in assay technologies have facilitated the development of high-throughput screening (HTS) (Major 1995; Pazhanisamy et al. 1995; Devlin 1997; Harding et al. 1997; Houston and Banks 1997; Sittampalam et al. 1997; Su et al. 1997; Brandt 1998; Fernandes 1998; González and Negulescu 1998; Kenny et al. 1998; Pasini et al. 1998; Sills 1998; Silverman et al. 1998; Wingfield 1998; Zysk and Baumbach 1998; Bolger 1999; Labute 1999; Winkler et al. 1999; Bosse et al. 2000; Cox et al. 2000; Feiglin et al. 2000; Gauglitz 2000; Landro et al. 2000; Meza 2000; Parker et al. 2000; Schuster et al. 2000; Sundberg 2000; Liu et al. 2004).

Zehender et al. (2004) recommended SpeedScreen as the “missing link” between genomics and lead discovery. SpeedScreen is a label-free, in-solution, affinity-based selection methodology for HTS. The protocol compromises in-solution affinity selection, followed by size exclusion chromatography in combination with microbore-liquid-chromatography/electrospray-ionization mass spectroscopy.

A versatile platform for nuclear receptor screening using AlphaScreen™ has been developed by Rouleau et al. (2003)

Abraham et al. (2004) discussed high-content screening applied to large-scale cell biology.

In **multiplexing assays**, more than one component is studied in a single sample. Advantages of this strategy include economy, since theoretically the same sample may be used for many measurements (Beske and Goldbard 2002). Also, because the exact same sample is used for different assays, sample errors caused by heterogeneity are minimized (Charter 2004). Nieuwenhuijsen et al. (2003) published a dual luciferase multiplexed HTS platform for protein–protein interactions. Maley et al. (2004) used multiplexed real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for HTS applications.

Grépin (2004) recommended multiplexed cell-based assays as an intermediary screening format between HTS and high content screening.

0.4.3.1

Dispensing Technologies

Several systems for fluid dispensing are in use: the piezo- and inkjet, the “air displacement”, and the “pintool” systems (Vollert et al. 2000). Piezoelectric and inkjet systems were the first introduced to the market (Lemmo et al. 1998; Rose 1999). There are three types of inkjet dispensers: thermal, solenoid, and piezoelectric. All of these use some means of compressing a liquid against a small orifice to create sufficient linear velocity to eject the fluid in the form of a drop.

The “pintool” systems use pins (needles) to transfer liquid. Up to 1536 needles are immersed into the fluid and withdrawn, taking up a few nanoliters and transferring the fluid into the wells of a microtiter plate.

Utilization of syringes and syringe needles as a contact tool enables multiple dispensing from a single fill. Generally, syringe dispensing has a higher degree of accuracy and repeatability compared with pin dispensing tools (Vollert 1998; Dunn and Feygin 2000).

Microfluidic arrays for high-throughput submicroliter assays using capillary electrophoresis were described by Gibbons (2000). Capillary electrophoresis is an analytical technique that can achieve rapid high-resolution separation of water-soluble components present in small sample volumes. Separations are based on the electrically driven movement of ions. In HTS, analytes are separated according to differences in electrophoretic mobility, depending on the charge-to-size ratio.

The most common “no contact” delivery format is piezoelectric acoustic delivery. This technique delivers a powerful sonic or ultrasonic pulse, which accel-

erates a tiny droplet from the surface of liquid in the stock vessel, through the air and onto the dry capture of a microplate. Hsieh et al. (2004) used disposable piezoelectric ejectors for liquid dispensing in ultra-high-throughput microarray screening.

0.4.3.2

Cell-Based Assays

Cell-based assays are increasingly attractive alternatives to *in vitro* biochemical assays for HTS (Wallace and Goldman 1997; Silverman et al. 1998). They fall into three broad categories: second messenger assays that monitor signal transduction following activation of cell-surface receptors; reporter gene assays that monitor cellular responses at the transcription/translation level; and cell proliferation assays to monitor the overall growth/no growth response of cells to external stimuli (Sundberg 2000).

Hynes et al. (2003) described a luminescence-based assay for screening the viability of mammalian cells, based on the monitoring of cell respiration by means of a phosphorescent water-soluble oxygen probe that responds to changes in the concentration of dissolved oxygen by changing its emission intensity and lifetime.

Yan et al. (2002) described a cell-based HTS assay system for monitoring G-protein-coupled receptor activation using β -galactosidase enzyme complementation technology. This technology uses a pair of inactive β -galactosidase deletion mutants as fusion partners to the protein targets of interest. To monitor G-protein-coupled receptor activation, stable cell lines expressing both G-protein-coupled receptor and β -arrestin- β -galactosidase fusion proteins are generated. Following ligand stimulation, β -arrestin binds to the activated G-protein-coupled receptor, and this interaction drives functional complementation of the β -galactosidase mutant fragments. G-protein-coupled receptor activation is measured directly by quantitating restored β -galactosidase activity with the chemoluminescent Gal-Screen reagent.

Cell-based reporter assays are used where human receptors are transfected into null cell lines either alone, or as part of receptor systems constructed to show alterations in light production (luciferin-luciferase) or light transmission (melanophore), which can be measured independently of radioactivity within minutes (Broach and Thorner 1996).

Pathirna et al. (1995) developed a transient transfection HTS assay to identify nonsteroidal human progesterone receptor modulators using luciferase expression. Zhu et al. (2004) found a good correlation be-

tween high-throughput pregnane X receptor (PXR) transactivation and binding assays.

Another reporter gene, green fluorescent protein, can be measured by fluorescence techniques in real time so that kinetic parameters can be determined from a single well of a microtiter plate (Chalfie et al. 1994; Chalfie 1995; Kain 1999; Meng et al. 2000; Arun et al. 2005).

Scheirer (1997) reviewed the various reporter genes and their applications.

Ting et al. (2001) described genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells.

González and Negulescu (1998) described intracellular detection assays for HTS which employ either fluorescent or luminescent readouts. The method is particularly suitable for measuring calcium mobilization (Akong et al. 1995; Burbaum and Sigal 1997).

Using receptor selection and amplification technology (R-SAT), it is possible to rapidly quantify specific pharmacological responses as a function of changes in cell proliferation rates (Messier et al. 1995). NIH3T3 cells (or other cell lines) are transfected with the plasmid coding for the receptor of interest and pSV- β -Gal. Cells are then transferred to 96-well microtiter plates and test compounds are added. Several days later, β -Gal activity is measured via a simple, inexpensive colorimetric assay. As a result of compound treatment, the changes in enzyme activity are a function of the proliferative activity of the cells. This functional assay can quantitatively differentiate between full agonists, partial agonists, neutral antagonists, and negative antagonists (inverse agonists). An interesting aspect of this technology is that many genes in a related family can be combined in the same cell population for convenient assay.

Bailey et al. (2002) discussed the applications of transfected cell microarrays in high-throughput drug discovery. Transfected cell microarrays are a complementary technique in which array features comprise clusters of cells overexpressing defined cDNAs. Complementary DNAs cloned in expression vectors are printed on microscope slides, which become living arrays after the addition of a lipid transfection reagent and adherent mammalian cells.

Dupriez et al. (2002) and Le-Poul et al. (2002) described aequorin-based functional assays for G-protein-coupled receptors, ion channels, and tyrosine kinase receptors.

AequoScreen is a cellular-based functional assay in which the cells are loaded with the apoaequorin cofac-

tor coelenterazine, diluted in assay buffer, and injected into plates containing the samples to be tested.

Kunz-Schughart et al. (2004) recommended the multicellular spheroid model and the use of 3-D cultures for HTS.

Gaspari et al. (2004) described the quantification of the proliferation index of human dermal fibroblast cultures with the ArrayScan™ high-content screening reader.

Isailovic et al. (2005) presented a high-throughput method for measuring single-cell fluorescence spectra. Upon excitation with a 488 nm argon-ion laser many bacterial cells were imaged by a 20 \times microscope objective while they moved through a capillary tube. Fluorescence was dispersed by a transmission diffraction grating, and an intensified charge-coupled device (ICCD) camera simultaneously recorded the zero and the first orders of the fluorescence from each cell.

Flow cytometry was recommended for high-throughput, high-content screening by Edwards et al. (2004). Flow cytometry is a platform for quantitative multi-parameter measurements of cell fluorescence. Homogeneous discrimination between free and cell-bound fluorescent probe eliminates wash steps to streamline sample processing. Waller et al. (2004) used flow cytometry as a technique for studying GPCR assembly, pharmacology, and screening.

0.4.3.3 High-Throughput Assay Technologies for Ion Channels

Ion channels represent a class of membrane-spanning protein pores that mediate the flux of ions in a variety of cell types. To date, >400 ion channels have been cloned and characterized, and some of these channels have emerged as attractive drug targets. Zheng et al. (2004b) reviewed the technologies that are currently used for the screening of ion channels. The technologies discussed are binding assays, ion flux assays, fluorescence-based assays, and automated patch-clamp instrumentation.

Gill et al. (2003) described flux assays in HTS of ion channels in drug discovery. Technologies based on flux assays are available in a fully automated high-throughput format for efficient screening. This application offers sensitive, precise, and reproducible measurements giving accurate drug rank orders matching those of patch-clamp data.

Treherne (2006) reviewed high-throughput ion channel screening technologies in integrated drug discovery.

Automated patch-clamp systems useful for HTS techniques, such as Qpatch, have been recommended by several authors (Asmild et al. 2003; Lepple-Wienhues et al. 2003; Stett et al. 2004; Willumsen et al. 2003; Brueggemann et al. 2004; Shieh 2004; Zheng et al. 2004b; Mathes 2006).

0.4.3.4

Detection Methods

Fluorescence-based assay technologies play an increasing role in HTS. When compared to other biochemical and cell-based techniques, fluorescence has a significant advantage over such methods as isotopic labeling, colorimetry, and chemiluminescence.

Sensitivity

It is relatively simple for modern instrumentation to reliably detect a signal being emitted from a single fluorescent molecule. In biological applications, this level of sensitivity means molecules that may be present only in very small numbers can be easily detected. In the case of imaging application, such as high-content screening, their intracellular location can be determined. This high level of sensitivity also means that transient biological events can be detected very quickly, hence enabling the measurement and understanding of events that occur very rapidly inside living cells. The inherent sensitivity of fluorescence technology also permits the use of very low concentrations of fluorescent label.

Specificity

Fluorescent probes are readily available for labeling virtually any biomolecule, structure, or cell type. Immunofluorescent probes can be directed to bind not only to specific proteins but also to specific conformations, cleavage products, or site modifications, such as phosphorylation. Individual peptides and proteins can be engineered to autofluorescence by expressing them as AFP chimeras inside cells. Such high levels of specificity enable the use of several different fluorescent labels – each emitting at a unique color – and the subsequent understanding of the complex interactions that occur among and between subcellular constituents.

Accuracy

Reagents used for fluorescence labeling are well understood and their performance has been characterized under a wide variety of biological environments.

Flexibility

Fluorescence technologies have matured to the point where an abundance of useful dyes is now commercially available. Many dyes have been developed and optimized for labeling specific cell compartments and components, as well as for labeling proteins (antibodies, ligands, toxins, etc.), which can be used as probes. Other fluorescent sensors have been designed to report on biological activities or environmental changes (pH, calcium concentration, electrical potential, proximity to other probes, etc.). Multiple fluorescent labels can be used on the same sample and individually detected quantitatively, permitting measurement of multiple cellular responses simultaneously. Many quantitative techniques have been developed to harness unique properties of fluorescence, including:

- fluorescence resonance energy transfer (FRET)
- fluorescence polarization or anisotropy (FP)
- time-resolved fluorescence (TRF)
- fluorescence lifetime measurements (FLM)
- fluorescence correlation spectroscopy (FCS)
- fluorescence photobleaching recovery (FPR)
- molecular beacons (MB)

Mere et al. (1999) reported miniaturized **fluorescence resonance energy transfer (FRET)** assays and microfluidics as key components for ultra-HTS. An ultra-HTS system (UHTSS™) platform integrates several microfluidic dispensing devices, which have been developed to deliver nanoliter to microliter volumes at high speed into the appropriate wells of 384-well NanoWell™ assay plates. The activity of the compounds dispensed into the assay wells is quantified via a highly sensitive dual-wavelength emission fluorescence detector.

Pope et al. (1999) discussed the advantages and disadvantages/limitations of fluorescence techniques for the establishment of miniaturized homogeneous screening assays, including prompt intensity assay (FLINT), anisotropy assay (FA), prompt energy transfer assay (FRET), fluorescence correlation spectroscopy (mass-dependent FCS), and fluorescent correlation spectroscopy (mass-independent FCS).

Nagai et al. (2004) used FRET technology to develop genetically encoded fluorescent indicators for various cell functions.

Chin et al. (2003) described miniaturization of cell-based beta-lactamase-dependent FRET assays to ultra-high-throughput formats to identify agonists of human liver X receptors.

Wallrabe and Periasami (2005) reviewed imaging protein molecules using Förster (or fluorescence) resonance energy transfer (FRET) and fluorescence lifetime imaging measurements (**FLIM**).

Dynamics of fluorescence fluctuations in green fluorescent protein observed by **fluorescence correlation spectroscopy (FCS)** were described by Haupts et al. (1998). Auer et al. (1999) recommended fluorescence correlation spectroscopy for lead discovery by miniaturized HTS. By monitoring interactions “of single molecules in femtoliter volumes, fluorescence correlation spectroscopy offers the highest potential as the detection technique at the nanoscale. Schwille et al. (2000) reported that fluorescence correlation spectroscopy reveals fast optical excitation-driven intramolecular dynamics of yellow fluorescent proteins.

Haupts et al. (2000) compared macroscopic versus microscopic fluorescence techniques in (ultra)-HTS.

Rüdiger et al. (2001) reviewed single-molecule detection technologies in miniaturized HTS and studied binding assays for G-protein-coupled receptors using fluorescence intensity distribution analysis (**FIDA**) and fluorescence anisotropy. As a confocal detection technology, FIDA inherently allows reduction of the assay volume to the microliter range and below without any loss of signal.

Palo et al. (2002) introduced a method that combines the benefits of both fluorescence intensity distribution analysis (**FIDA**) and fluorescence lifetime analysis. It is based on fitting the two-dimensional histogram of the number of photons detected in counting time intervals of given width and the sum of excitation to detection delay times of these photons. Referred to as fluorescence intensity and lifetime distribution analysis (**FILDA**), the technique distinguishes fluorescence species on the basis of both their specific molecular brightness and the lifetime of the excited state and is also able to determine absolute fluorophore concentrations.

Jäger et al. (2003a) reviewed fluorescence techniques for high-throughput drug discovery. In particular, the family of fluorescence techniques, Fluorescence Intensity Distribution Analysis (FIDA), which is based on confocal single-molecule detection, has opened up a new field of HTS applications.

Egging et al. (2005) recommended the combination of Förster resonance energy transfer (FRET) and two-color global fluorescence correlation spectroscopy (2CG-FCS) for high-throughput applications in drug screening.

Sportsman and Leytes (2000) discussed miniaturization of homogenous assays using **fluorescence po-**

larization (FP), which can detect changes in molecule size as well as quantify the binding of small molecules to larger molecules. Polarization of fluorescence occurs when a fluorescent molecule is illuminated with plane-polarized light, provided that the molecule does not move during the course of fluorescent lifetime. For typical fluorescent molecules such as fluorescein, the polarization is not observed if the molecule is rotating rapidly in solution under conditions typically used in biological assays. Thus, if the polarization of fluorescence of a fluorescein-labeled ligand is measured, a low polarization will be observed when the ligand is free in solution, and a high polarization will be observed when the ligand is bound to a macromolecule such as a specific receptor or antibody.

Banks et al. (2000) described fluorescence polarization assays for HTS of G-protein-coupled receptors in 384-well microtiter plates.

Parker et al. (2000) used fluorescence polarization to develop HTS assays for nuclear receptor-displacement and kinase inhibition. This method is a solution-based, homogeneous technique, requiring no immobilization or separation of reaction components. As an example, the authors described the fluorescence polarization-based estrogen receptor assay based on the competition of fluorescein-labeled estradiol and estrogen-like compounds for binding to the estrogen receptor.

Li et al. (2000) developed an assay for measuring the activity of an enzyme that transfers multiple adenine-containing groups to an acceptor protein, based on fluorescence polarization technology in a 1536-well plate format. Texas red (rhodamine) was covalently conjugated to the adenine of the donor substrate through a C₆ spacer arm. As a result of the transfer of the adenine-containing moieties to the acceptor protein substrate, the rotational correlation time of the Texas red conjugate increased, hence increasing the degree of fluorescence polarization.

Owicki (2000) compared the perspectives of fluorescence polarization and anisotropy in HTS.

Qian et al. (2004) used fluorescence polarization to discover novel inhibitors of Bcl-xL, a factor preventing apoptosis.

Lu et al. (2004) developed a fluorescence polarization bead-based coupled assay to target activity/conformation states of a protein kinase. This assay is based on the principle of the Immobilized Metal Assay for Phosphochemicals (IMAP™) (Mole™mular Devices, Sunnyvale, Calif., USA), a fluorescence polarization assay based on the affinity capture of phosphorylated peptides (Gaudet et al. 2003; Sportsman et al. 2003).

Kolb et al. (1997) and Grépin and Pernelle (2000) described the evolution of **homogeneous time resolved fluorescence (HTRF)** technology for HTS. HTRF uses the europium (Eu^{3+}) ion caged into polycyclic cryptate (Eu -cryptate) as a donor. Laser excitation at 337 nm transfers the energy from the Eu -cryptate complex to an allophycocyanin acceptor molecule (XL665), the APC. This results in the emission of light at 665 nm over a prolonged time (milliseconds). Uncoupled or free XL665 emits at 665 nm, but with a short decay. The light emission is recorded in a time-resolved fashion over a 400- μs period, starting 50 μs after the excitation pulse, so that the autofluorescence from the media and the short-lived fluorescence of the free APC are not recorded. Eu -cryptate emits at 620 nm and is discriminated from XL665 by wavelength.

The labeling of macromolecules for HTRF assay can be done in a number of ways, depending on the type of screen, using direct, indirect or semi-direct types of labeling. Direct labeling is characterized by having the (Eu)K and XL665 covalently bound to the molecule or molecules of interest. Indirect labeling is when the (Eu)K and XL665 are bound to macromolecules through secondary interactions, such as biotin-streptavidin interaction or antibody binding. Other indirect methods, such as (Eu)K or XL665-labeled lectin, can be used for binding to membranes. The semi-direct method is a combination of direct and indirect methods and is frequently used in HTRF.

HTRF immunoassays generally use a direct labeling strategy in which two antibodies against different antigenic sites are used. One antibody is labeled with (Eu)K and another is labeled with XL665. When both bind to the antigen, energy transfer occurs and the long-lived XL665 signal is generated. Europium-labeled recombinant protein G as a fast and sensitive immunoreagent for time-resolved immunofluorometry had been described by Markela et al. (1993).

Merk et al. (2004) described time-resolved fluorescence measurements using a microlens array and area imaging devices. For applications in the TRF mode the Plate::Vision(2) 96-microlens array reader (Carl Zeiss Jena, Germany) was compared with the LEADseeker Generation IV multimodality imaging system (Amersham Biosciences UK).

Selvin (2002) discussed the principles and biophysical applications of lanthanide-based probes. Using luminescent lanthanides, instead of conventional fluorophores, as donor molecules in resonance energy transfer measurements offers many technical advantages and opens up a wide range of applications includ-

ing farther measurable distances with greater accuracy, insensitivity to incomplete labeling, and the ability to use generic relatively large labels.

Karvinen et al. (2002) developed a homogenous time-resolved fluorescence quenching assay (LANCE) for caspases. The assay uses a peptide labeled with both a luminescent europium chelate and a quencher. Cleavage of the peptide by caspase-3 separates the quencher from the chelate and thus recovers europium fluorescence.

To study the interaction of two proteins, the JUN FOS dimerization was used as a model assay. The JUN protein was biotinylated. Streptavidin:(Eu)K was used to bind that label to the JUN-biotin protein via SA-biotin binding. The FOS peptide was directly labeled with XL665. Signal is only generated when JUN-biotin:SA-(Eu)K and FOS-XL665 dimerize to form the activated AP1 transcription complex.

The binding of ligands to membrane receptors and the competitive displacement by unknown molecules is one of the most common screening assays in drug discovery. To test the applicability of HTRF for receptor binding, the interaction of epidermal growth factor (EGF) with its receptor (EGFR) was studied (Mathis et al. 1994). To generate a HTRF signal, a semi-direct labeling method was developed. EGF was labeled directly with (Eu)K, and a monoclonal antibody against a nonbinding portion of the EGF receptor was used. The anti-EGFR antibody was labeled with XL665. The IC_{50} of displacement could be determined.

The phosphorylation of enzymes, proteins and receptors is a major mechanism of cell regulatory pathways and is therefore a frequent target for drug discovery. The EGFR-intrinsic tyrosine kinase activity was used to develop an HTRF assay. Biotin-labeled (glutamine-alanine-tyrosine)_n (poly GAT) was used as a substrate. The EGF receptor was isolated from A431 cells after partial purification of a cell homogenate. After the phosphorylation reaction, the HTRF signal was generated by adding streptavidin-XL665 and (Eu)-K-antiphosphotyrosine antibody. To establish the biological function of HTRF in this assay, inhibition of tryphostin-47 was assayed.

Homogeneous **time-resolved fluorescence resonance energy transfer (TR-FRET) assays** represent a highly sensitive and robust HTS method for the quantification of kinase activity (Lundin et al. 2001). Moshinski et al. (2003) reported a widely applicable high-throughput TR-FRET assay for the measurement of kinase autophosphorylation. Inhibition of the vascular endothelial growth factor receptor 2 (VEGFR-2) was used as prototype.

Glickman et al. (2002) published a comparison of ALPHAScreen, TR-FRET and TRF as assay methods for FXR nuclear receptors.

Newman and Josiah (2004) described utilization of fluorescence polarization and time-resolved fluorescence resonance energy transfer assay formats for structure–activity relationship studies. Src kinase was used as a model system.

Beasley et al. (2003) described the evaluation of compound interference in immobilized metal ion affinity-based fluorescence polarization (IMAP) detection with a four million member compound collection. IMAP is a non-separation-based, antibody-independent FP assay that can be applied to many types of protein kinases and phosphatases. In this technology, a fluorescently labeled peptide substrate is phosphorylated and then captured on immobilized metal nanoparticles. The binding of the phosphorylated peptide to the nanoparticles is detected using FP.

Loomans et al. (2003) reported HTS with immobilized metal ion affinity-based fluorescence polarization detection as a homogeneous assay for protein kinases.

Klumpp et al. (2006) discussed the development of homogeneous, miniaturized assays for the identification of novel kinase inhibitors from very large compound collections. In particular, the suitability of TR-FRET based on phospho-specific antibodies, an antibody-independent fluorescence polarization (FP) approach using metal-coated beads (IMAP technology), and the determination of adenosine triphosphate consumption through chemiluminescence was evaluated.

The **Acumen Explorer** is a laser-scanning system that measures fluorescence and luminescence, combining the ability to detect sub-micron events and features with ultra-fast acquisition and data management. Different parts of the cell can be localized by labeling with Hoechst 33342, AlexaTM 488, AlexaTM 546, AlexaTM 594, or CysTM 5. Many different colors (readouts) can be used simultaneously.

The **fluorometric imaging plate reader (FLIPRTM)** permits rapid, kinetic measurements of intracellular fluorescence. Simultaneous measurements in 96 wells and in real time can be made every second: before, during and after the addition of test compounds. The detection optics of FLIPR are based on cooled charge-coupled device (CCD) technology. With each kinetic update, the system takes a picture of the bottom of a microplate, recording a signal for all the individual cells simultaneously. Enhanced sensitivity for cell-based assays is accomplished via an optical detection scheme, which allows for signal isolation on a cell monolayer. The system is ideal for monitoring

intracellular calcium fluxes that occur within seconds of activation of G-coupled receptors. Using calcium-sensitive dyes such as Fluo-3 permits the derivation of full dose–response (or dose–inhibition) curves in a matter of minutes (Kuntzweiler et al. 1998; Coward et al. 1999; Miller et al. 1999; Milligan and Rees 1999; Sullivan et al. 1999).

Hodder et al. (2004) reported miniaturization of intracellular calcium functional assays to the 1536-well plate format using a fluorometric imaging plate reader. An intracellular calcium functional assay against the rat muscarinic acetylcholine receptor subtype 1 G-protein-coupled receptor was miniaturized and executed in modified instruments.

Robinson et al. (2004) used a fluorescence microplate reader to compare ratiometric and nonratiometric Ca²⁺ indicators for the assessment of intracellular free Ca²⁺ in a breast cancer line.

Bednar et al. (2004) used fluorescence detection of calcium flux for kinetic characterization of novel NR2B antagonists.

Swartzman et al. (1999) developed a simple, homogeneous bead-based immunoassay for use with **fluorometric microvolume assay technology (FMAT)**.

Koltermann et al. (1998) proposed rapid assay processing by integration of **dual-color fluorescence cross-correlation spectroscopy (RAPID FCS)** as an ideal tool for ultra-HTS when combined with nanotechnology, which can probe up to 10⁵ samples per day. Auer et al. (1999) used fluorescence correlation spectroscopy for lead discovery by miniaturized HTS.

Winkler et al. (1999) presented **confocal fluorescence coincidence analysis** as an alternative to HTS. Confocal fluorescence coincidence analysis extracts fluorescence fluctuations that occur coincidentally in two different spectral ranges from a tiny observation volume of less than 1 fl. This procedure makes it possible to monitor whether an association between molecular fragments labeled with different fluorophores is established or broken, providing access to the characterization of a variety of cleavage and ligation reactions in biochemistry.

Jäger et al. (2003b) published a modular, fully integrated ultra-HTS system based on **confocal fluorescence analysis techniques**. The goal was to achieve high-data quality in small assay volumes (1–4 µl) combined with reliable and unattended operation. Two new confocal fluorescence readers have been designed. One of the instruments is a four-channel confocal fluorescence reader, measuring with four objectives in parallel. The fluorescence readout is based on single-molecule detection methods, allowing high sensi-

tivity at low tracer concentrations and delivering an information-rich output. The other instrument is a confocal fluorescence imaging reader, where the images are analyzed in terms of generic patterns and quantified in units of intensity per pixel. Both readers span the application range from assays with isolated targets in homogenous solution or membrane vesicle-based assays (four-channel reader) to cell-based assays (imaging reader). Results from a comprehensive test on these assay types demonstrate the high quality and robustness of this screening system.

Heilker et al. (2005) recommended confocal fluorescence microscopy for HTS of G-protein-coupled receptors.

For HTS, Schmid et al. (1998) proposed a method to reversibly attach receptor proteins via an affinity tag to a quartz surface and subsequently detect with high sensitivity the real-time binding of ligands by **total internal reflection fluorescence**.

The **CytoFluor Fluorescence Multi-Well Plate Reader** is a versatile fluorescence intensity measurement instrument that detects and quantifies fluorescent molecules over a five-log dynamic range. The system has a sensitivity of better than 8 fmol/well for fluorescein.

Ramm (1999) described the technique of **image-based screening in the LEADseeker™ system**, which contains a cooled CCD camera and a telecentric lens with integral epifluorescence illumination.

Laser scanning imaging systems have been developed to measure cellular and subcellular quantitation of fluorescence in whole cells (Conway et al. 1999; Zuck et al. 1999; Hertzberg and Pope 2000).

Chemiluminescence is a photometric technique that is applicable to HTS. Detection of chemiluminescence is a convenient adjunct to fluorescence, since most plate readers capable of measuring fluorescence will measure luminescence as well. This technique has been used predominantly with luciferase reporter genes in cell-based assays and in high-sensitivity enzyme-linked immunosorbent assays (ELISA), employing chemolumogenic substrates for alkaline phosphatase and horseradish peroxidase (Bronstein et al. 1994, 1996). The Luc-Screen assay system with extended-glow light emission was designed for sensitive detection of the firefly luciferase reporter enzyme. Liochev and Fridovich (1997) recommended lucigenin luminescence as a measure of intracellular superoxide dismutase activity in *Escherichia coli*.

The NorthStar™ HTS workstation has been developed as a chemiluminescence analyzer for the HTS laboratory. Featuring online injection, a broad dynamic

range, and an extremely low crosstalk, the NorthStar™ HTS workstation allows the routine analysis of more than 500,000 samples a day.

Grépin et al. (2001) presented an assay for precise and direct quantification of specific endogenous mRNAs in cell lysates. The technology is based on the bioluminescence detection of multiple biotinylated ssDNA probe/endogenous mRNA hybrids, which are captured onto a streptavidin-coated multi-well plate. Using the Xpress-Screen™ technology, an assay was developed aimed to monitor the induction of endogenous cFos mRNA expression in NGF-treated PC12 cells and the miniaturization of the assay in a 384-well format, which is adapted to HTS.

Pommereau et al. (2004) compared the performance of two HTS assays for a serine/tyrosin kinase: a microplate-based, bioluminescent assay that uses the luciferin/luciferase system to monitor ATP consumption, and a microfluidic assay that measures the change in mobility in an electric field of a fluorescently labeled peptide upon phosphorylation.

O'Brien et al. (2005) described homogenous, bioluminescent protease assays using caspases-3 as a model that are significantly faster and more sensitive than the fluorescent caspases-3 assays.

David et al. (2002), Gopalakrishnan et al. (2002), and Anderson et al. (2004) used **microarrayed compound screening (μARCS)**, a well-less, high-density, ultra-HTS format, for various assays. In μARCS, the reagents are incorporated into agarose gels and layered over compounds arrayed on polystyrene sheets, mimicking the pipetting steps in a microplate assay. μARCS is a very flexible format and eliminates the requirement for complex liquid handling instruments and avoids problems associated with evaporation and plate edge effects.

Bioluminescence resonance energy transfer (BRET) was developed as a technique to study protein-protein interactions. BRET is a natural phenomenon, observed in marine organisms, in which energy transfer occurs between luminescent donor and fluorescent acceptor proteins. Oxidation of coelenterazine by *Renilla* luciferase produces light with a wavelength of 480 nm. In the sea pansy *Renilla*, the close proximity of a green fluorescent protein allows a non-radiative energy transfer that results in light emission at 509 nm by the green fluorescent protein. In the BRET technology, the first protein partner is fused to *Renilla* luciferase, whereas the second protein is fused to a fluorescent protein, e. g., yellow fluorescent protein. If the two partners do not interact, only one signal, emitted by the luciferase, can be

detected after addition of its substrate coelenterazine. If the distance between the two partners is less than 10 nm (100 Å) and the partners interact, resonance energy transfer occurs between the luciferase and the yellow fluorescent protein and an additional signal can be detected. The method was used to study the dimerization of G-protein-coupled receptors (Angers et al. 2000, 2001; Cheng and Miller 2001; Kroeger et al. 2001; McVey et al. 2001; Ayoub et al. 2002). Based on BRET technology, Boute et al. (2002) developed a test to monitor the activation state of the insulin receptor. BRET technology was recommended for HTS assays.

A BRET2 assay was recommended by Bertrand et al. (2002). *Renilla* luciferase was used as the donor protein, while the green fluorescent protein 2 was used as the acceptor protein. In the presence of the cell-permeable substrate DeepBlueC, *Renilla* luciferase emits blue light at 395 nm. If the green fluorescent protein 2 is brought into close proximity to *Renilla* luciferase via a specific biomolecular interaction, it will absorb the blue light energy and re-emit green light at 510 nm.

Vrecl et al. (2004) described the development of a BRET2 screening assay using β -arrestin 2 mutants.

Pfleger and Eidne (2004) used BRET for the detection of real-time interactions involving G-protein-coupled receptors.

Heding (2004) described the BRET 7TM receptor/beta-arrestin assay in drug discovery and screening that can be used to screen all seven-transmembrane receptors independent of their signaling pathway.

Electrochemiluminescence has been recommended as a diagnostic and research tool (Yang et al. 1994). Electrochemiluminescence is a means of converting electrical energy into radiative energy. It involves the production of reactive intermediates from stable precursors at the surface of an electrode. These intermediates then react under various conditions to form excited states that emit light (Richter 2003).

Golla and Seethal (2004) reported a sensitive, robust, high-throughput electrochemiluminescence assay for rat insulin.

The introduction of the **scintillation proximity assay (SPA)** obviated the need to separate bound from free radioactivity in the conventional radioligand-binding assay (Bosworth and Towers 1989; Picardo and Hughes 1997; Game et al. 1998; Wu and Liu 2005). One version of the scintillation proximity assay utilized polyvinyltoluene microspheres or beads into which a scintillant has been incorporated. When a radi-

olabeled ligand is captured on the surface of the bead, the radioactive decay occurs in close proximity to the bead, and effectively transfers energy to the scintillant, which results in light emission (Sittampalam et al. 1997).

Zheng et al. (2004a) reported high-throughput cell-based screening using the scintillation proximity assay for the discovery of inositol phosphatase inhibitors.

FlashPlateTM technology has been described by Brown et al. (1997). FlashPlates are white 96-well polystyrene microplates coated on the inside with solid scintillant in a polystyrene polymer. The polystyrene surface provides a hydrophobic surface for absorption of proteins, such as antibodies and receptors. When the binder molecule is coated on the FlashPlate, the reaction of the mixture of radiolabeled tracer and unlabeled ligand of interest with the binder molecule, and detection of the bound radioactivity can be done in one step. Due to the microplate surface scintillation effect, only the bound radioactivity will be detected by a scintillation counter.

Dillon et al. (2003) used a FlashPlate assay for the identification of PARP-1 inhibitors.

The use of **high-performance microphysiometry** in drug discovery has been discussed by Alajoki et al. (1997). Microphysiometry measures the extracellular acidification rate. It is governed by catabolism, via the excretion of acidic end-products, such as carbon dioxide and lactic acid. In addition, it is substantially influenced in a complementary fashion by the regulation of intracellular pH, a process sensitive to physiological changes such as receptor activation (see also Sects. A.1.1.7 and E.5.1.2). The light-addressable potentiometric sensor (LAPS) uses a light-generated alternating current to probe the electrical potential at the interface between an electrolyte and the insulated surface of an appropriately biased silicon chip; the surface potential depends on pH in a Nernstian fashion. This system has been optimized for HTS.

Microarray compound screening (μ ARCS) was used by David et al. (2002), Gopalakrishnan et al. (2002), and Anderson et al. (2004).

Abassi et al. (2004), Atienza et al. (2005), and Yu et al. (2006) described dynamic monitoring of cell adhesion and spreading and real-time monitoring of morphological changes in living cells on **microelectronic sensor arrays**. Special microplates are integrated with electronic sensor arrays.

A promising probe for quantitative genomic studies is the **molecular beacon (MB)**, a single-stranded DNA molecule composed of a hairpin-shaped oligonucleotide that contains both a fluorophore and

a quencher group. Molecular beacons act like switches that are normally closed – when the fluorophore and the quencher are together, fluorescence is “off”. Conformational changes open the hairpin, separate the fluorophore and the quencher, and the fluorescence is turned “on”. These sensitive fluorescent DNA probes can be used for real-time biomolecular recognition of unseparated target species (Fang et al. 2000). Molecular beacons are useful for studies with proteins, molecular beacon aptamers (Yamamoto and Kumar 2000; Hamaguchi et al. 2001; Tan et al. 2004). Santangelo et al. (2004) described dual FRET molecular beacons for mRNA detection in living cells.

Surface plasmon resonance (Myszka and Rich 200; McDonnell 2001) is a method for characterizing macromolecular interactions. It is an optical technique that uses the evanescent wave phenomenon to measure changes in refractive index very close to a sensor surface. The binding between an analyte in solution and its ligand immobilized on the sensor surface results in a change in the refractive index. The interaction is monitored in real time and the amount of bound ligand and rates of association and dissociation can be measured with high precision. Since there is no need for fluorescent or radioisotopic labels, surface plasmon resonance biosensors are amenable to characterizing unmodified biopharmaceuticals, studying the interaction of drug candidates with macromolecular targets and identifying binding partners.

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2000; Steiner and Anderson 2000; Peet and Bey 2001; Winkelmann 2001, Hemmilä and Hurskainen 2002).

Genetic polymorphism in drug-metabolizing enzymes, transporters, receptors, and other drug targets are linked to individual differences in the efficacy and toxicity of many medications. Pharmacogenomic studies can elucidate the inherited nature of these differences, thereby enhancing the possibilities of drug discovery and providing a stronger scientific basis for optimizing drug therapy on the basis of each patient's genetic constitution (Evans and Relling 1999; Grant 2001).

Receptor polymorphism is also the reason for differences in drug effects among patients. Two genetic variants of the angiotensin-converting enzyme (ACE) are described, depending on an insertion (I-form) or deletion (D-form) of a base pair at position 287 in the gene, which are equally distributed in the Caucasian population. Individuals with the D/D-form express ACE levels much higher than I/I individuals. They have an increased risk for myocardial infarction and may respond better to therapy with ACE inhibitors (Samani et al. 1996; Danser and Schunkert 2000). Another example is the association between β_2 -adrenergic receptor polymorphisms and asthma (Liggett 1997). There are three polymorphisms that alter receptor function, which may influence the therapeutic success.

Genomics will introduce a new dimension in drug research. The number of molecular targets modified by the complete armamentarium of modern drugs is not greater than 500. The number of genes that contribute to multifactorial diseases might not be very high; the numbers reported for different forms of diabetes and hypertension are 5–10 per disease (Guillausseau et al. 1997; Shimkets and Lifton 1996). There are 100–150 disease entities that present an epidemiological and economical problem to industrialized societies. If one assumes 10 contributing genes for 100 multifactorial diseases (including different forms of cancer, asthma, diabetes, hypertension, atherosclerosis and osteoporosis), one arrives at 1000 “disease genes” that dispose patients to the most important multigenetic conditions (Drews 2000, 2001). It appears reasonable to assume that each of these disease genes, or rather proteins that are specified by the disease genes, connects with at least 5–10 proteins that are feasible levels for drug intervention. On the basis of these calculations, one can assume that 5000–10,000 can be used as targets for drug interventions.

Lennon (2000) discussed the methods for high-throughput gene expression analysis applicable to drug

discovery, including differential display of eukaryotic mRNA, the modification thereof called restriction enzyme analysis of differentially expressed sequences (READS), expression sequence tags (EST) methodology, serial analysis of gene expression, filter arrays, and DNA microarrays.

Celis et al. (2000) gave an excellent survey on microarray technology.

Gene expression analyses and genetic polymorphisms are important not only for determining predisposition to disease and for drug discovery, but also for predicting the incidence of adverse drug reactions (Meyer 2000). In the past, several drugs had to be withdrawn from the market due to rare, but severe, adverse events. Some drug metabolic enzyme variants have been found to cause severe adverse events. They form qualitatively or quantitatively different metabolites with toxicological implications (Bullingham 2001).

Most of the enzymes involved in both drug metabolism and the elimination of many therapeutic agents are members of the cytochrome P450 (CYP) family that includes more than 30 isoforms (Gonzales 1990). One drug-metabolizing enzyme, resulting in numerous drugs being withdrawn from the market, is CYP3A, which is involved in the oxidative biotransformation of up to 50% of clinically important therapeutic agents. The expression of CYP3A is regulated by genetic and non-genetic factors that can result in a 5- to 20-fold interindividual variability in metabolic clearance. Another enzyme, CYP2D6 (debrisoquine hydroxylase), metabolizes one-quarter of all prescribed drugs and is inactive in 6% of the Caucasian population (Wolf and Smith 1999). A principal molecular defect in poor metabolizers is a single base pair mutation in exon 5 of CYP2C19 (De Morais et al. 1994; Sadée 1999). These enzymes are being screened at the earliest stages of the drug development process. The single nucleotide point mutation profile of an individual can indicate a predisposition for adverse side-effects. Furthermore, drug transport protein polymorphisms and their drug interactions may provide another valuable tool in screening for potential toxic effects of drugs. The pharmacogenomic approach should reduce the number of compounds failing during late clinical development (Adam et al. 2000).

A detailed knowledge of the genetic basis of individual drug response is of major clinical and economic importance and can provide the basis for a rational approach to drug prescription. In the sense of a “personalized medicine”, prospective genotyping will lead to patients being prescribed drugs that are both safer

and more effective (Sadée 1999; March 2000; Murphy 2000; Spear et al. 2001; Dietel and Sers 2006).

Winkelmann et al. (2001) initiated the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, which aims to provide a resource for the study of environmental and genetic risk factors and their interactions, and of the functional relationship between gene variation and biochemical phenotype (functional genomics), or response to medication (pharmacogenomics) and long-term prognosis of cardiovascular disease.

A vision for the future of genomics research was given by Collins et al. (2003).

Rolling-cycle amplification (RCA) is a system for amplification, which may be used for detection of proteins or nucleic acid targets (Lizardi et al. 1998; Zhong et al. 2001). Rolling-circle amplification driven by DNA polymerase can replicate circularized oligonucleotide probes with either linear or geometric kinetics under isothermal conditions. In the presence of two primers, one hybridizing to the positive strand and the other to the negative strand of DNA, a complex pattern of DNA strand displacement ensues that generates 10^9 or more copies of each circle in 90 min, enabling detection of point mutations in human genomic DNA. Using a single primer, RCA generates hundreds of tandemly linked copies of a covalently closed circle in a few minutes. I-matrix-associated, the DNA product remains bound to the site of synthesis, where it may be tagged, condensed, and imaged as a point light source. The target DNA can be attached to an antibody, biotin, or other protein probe.

Vanhecke and Janitz (2005) discussed functional genomics using high-throughput RNA interference. **RNA interference (RNAi)** describes the post-transcriptional silencing of gene expression that occurs in response to the introduction of double-stranded RNA into cells. To facilitate large-scale functional genomics studies using RNAi, several high-throughput approaches have been developed based on microarray or microwell assays. The RNAi silencing mechanism is based on the fact that long double-stranded RNA is processed to short interfering RNA (**siRNA**) duplexes by the enzyme Dicer, which exhibits RNase-III-like activity. A single strand of the siRNA is incorporated into the group of cytoplasmic proteins to form an RNA-induced silencing complex (**RISC**). Activated RISC, guided by the antisense siRNA strand, performs endonucleolytic cleavage of target mRNA. Thus produced mRNA fragments are rapidly degraded by cytoplasmic nucleases (Tijsterman and Plasterk 2004).

Rondinone (2006) discussed the therapeutic potential of RNAi in metabolic diseases.

Aza-Blanc et al. (2003) described the identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. TRAIL is a TNF superfamily member that induces selective cytotoxicity of tumor cells when bound to its cognate receptors (Lee et al. 2002).

Using RNAi, Kamath et al. (2003) performed a systematic functional analysis of the *Caenorhabditis elegans* genome. Mousses et al. (2003) describe a microarray analysis in cultured mammalian cells.

Paddison et al. (2004) described a resource for large-scale RNA-interference-based screens in mammals; Berns et al. (2004), a large-scale RNAi screen in human cells to identify new components of the p53 pathway.

Promega Protocols and Applications Guide is a published survey on RNA interference.

The use of siRNA in therapy and drug target discovery is discussed by Cocks and Theriault (2004), Hannon and Rossi (2004), Jones et al. (2004), Kurreck (2004), Meister and Tuschli (2004), Siuod (2004), Bartz and Jackson (2005), and Catterjee-Kishore (2006).

Xin et al. (2004) discussed high-throughput siRNA-based functional target validation. They initiated a project with a functional cell-based screen for a biological process of interest using libraries of small interfering RNA (siRNA) molecules. In this protocol, siRNAs function as potent gene-specific inhibitors. SiRNA-mediated knockdown of the target gene is confirmed by TaqMan analysis, and genes with impacts on biological functions of interest are selected for further analysis. Once the genes are confirmed and further validated, they may be used for HTS to yield lead compounds.

Tang (2005) discussed two classes of short RNA molecules, siRNA and microRNA (miRNA), which are incorporated into related RNA-induced silencing complexes (RISCs), termed siRISC and miRISC, respectively. They are distinct complexes that regulate mRNA stability and translation.

MicroRNAs (miRNAs) are generated via a two-step processing pathway to yield ~22 nucleotide small RNAs that regulate gene expression at the post-transcriptional level (Lee et al. 2002). Initial cleavage is catalyzed by Drosha, a nuclease of the RNase III family, which acts on primary miRNA transcripts (pri-miRNAs) in the nucleus (Lee et al. 2003).

MicroRNAs play important regulatory roles in animals and plants by targeting mRNAs for cleavage

or translational repression (Bartel 2004). The microRNAs of *Caenorhabditis elegans* are described by Lim et al. (2003).

Denli et al. (2004) showed that Drosha exists in a multi-protein complex, the microprocessor, and begins the process of destructing that complex into its constituent components. Along with Drosha, the microprocessors also contains Pasha (partner of Drosha), a double-stranded RNA binding protein. Han et al. (2004) dissected the mechanisms of action of human Drosha by generating mutants and by characterizing its interacting partner, DGCR8.

Berezikov et al. (2005) described phylogenetic shadowing and computational identification of human microRNA genes.

Barad et al. (2004) reported microRNA expression detected by oligonucleotide microarray system establishment and expression profiling in human tissue. Liang et al. (2005) described a miRNA profiling microarray, in which miRNAs are directly labeled at the 3' terminus with biotin and hybridized with complementary oligo-DNA probes immobilized on glass slides, and subsequently detected by measuring the fluorescence of quantum dots labeled with streptavidin bound to miRNAs through streptavidin-biotin interaction.

Locked nucleic acids (LNA) were reported in 1998 (Obidia et al. 1998; Koshkin et al. 1998). LNA is a nucleic acid analog containing one more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA-mimicking sugar confirmation. LNA nucleotides display unprecedented hybridization affinity toward complementary single-stranded RNA and complementary single- or double-stranded DNA (Braasch and Corey 2001; Vester and Wengel 2004). They are considered as a versatile tool for therapeutics and genomics (Petersen and Wengel 2003; Jepsen et al. 2004).

Tolstrup et al. (2003) reported the development of a software package, OligoDesign, which provides optimal design of LNA oligonucleotide capture probes for gene expression profiling.

Válóczi et al. (2004) described sensitive and specific detection of microRNAs by Northern blot analysis using LNA-modified oligonucleotide probes.

Elmén et al. (2005) reported LNA-mediated improvements in siRNA stability and functionality.

Thomassin et al. (2004) presented MethylQuant, a sensitive method for quantifying methylation of specific cytosines within the genome. The presence of a locked nucleic acid at the 3' end of the discriminative primer provides the specificity necessary for accurate and sensitive quantification.

Nucleic acid “**lariats**” have been of great interest since their discovery (Wallace and Edmonds 1983). The synthesis of lariat RNA and DNA via template-mediated chemical ligation of Y-shaped oligonucleotides was reported by Carriero and Damha (2003). Deoxyribozymes that synthesize branched and lariat RNA were studied by Wang and Silverman (2003). RNase III-mediated degradation of unspliced pre-mRNAs and lariat introns was reported by Danin-Kreisel et al. (2003). Coombes and Boeke (2005) evaluated the detection methods for large lariat RNAs.

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0.4.5 Proteomics

The term “proteome” was coined by Wilkins et al. (1995) to describe the protein complement of the genome. The term proteomics was first used to describe the complexity of proteins expressed in an organism using two-dimensional gel electrophoresis (2-DE) followed by quantitative analysis. 2-DE remains the highest resolution protein separation method available, but the ability to concentrate and identify low abundance proteins has always been an extremely difficult problem. Mass spectrometry (MS) has been an integral part of approaching this problem (de Hoog and Mann 2004; Lane 2005). Although improvements in 2-D gel technology have been made since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics (Patterson 2000). The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and the widespread introduction of mass spectrometers, capable of data-dependent ion selection for fragmentation (MS/MS) (i. e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying together the first technological advances. The mass spectrometer instruments are named for their type of ionization source and mass analyzer (Patterson and Abernold 1995; Carr and Annan 1997; Patterson 1998).

To measure the mass of molecules, the test material must be charged (ionized) and desolvated. The two most successful mechanisms for ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively efficient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer, that measures the elapsed time from acceleration of the charged (ionized) molecules through a field-free drift region (Kowalski and Stoerker 2000). The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC column or a nanospray device (Wilm and Mann 1994), similar to a microinjection needle. The ions formed during this process are directed into the mass analyzer, which could be a triple-quadrupole, an ion trap, a Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qp-TOF) type (Morris et al. 1996). Although unambiguous identification of a protein cannot always be derived from the masses of a few of its peptides in the tandem mass spectrometer, peptide ions from the first mass spectrometer run are fragmented and identified in a second run to yield a more valuable commodity of a peptide sequence.

Annis et al. (2004) and Whitehurst et al. (2006) proposed affinity selection-mass spectroscopy (AS-MS) as a useful high-throughput assay system for the discovery and characterization of all classes of integral membrane protein ligands, including allosteric modulators.

The goal of proteomics is a comprehensive, quantitative description of protein expression and its change under the influence of biological perturbations, such as disease and drug treatment (Anderson and Anderson 1998, 2002; Müller et al. 1998; Blackstock and Weir 1999; Dove 1999; Hatzimanikatis et al. 1999; Jungblut et al. 1999; Williams 1999). A combination of mRNA and protein expression patterns has to be simultaneously considered to develop a conceptual understanding of the functional architecture of genomes and gene networks (Kreider 2000). New methods are created such as automated proteomics platforms (Quadroni and James 1999; Nielsen et al. 1999), combining two-dimensional electrophoresis, automated spot picking and mass spectrometry (Binz et al. 1999; Dancik et al.

1999; Loo et al. 1999; Dutt and Lee 2000; Feng 2000; Patterson et al. 2000; Ryu and Nam 2000; Service 2000; Yates 2000; James 2001; Jain 2001; Rabilloud 2001).

Improvements in quality, ability and utility of large-scale tertiary and quarternary protein structural information are enabling a revolution in rational design, having a particular impact on drug discovery and optimization (Maggio and Ramnarayan 2001).

Disease proteomics will give a better understanding of disease processes, develop new biomarkers for diagnosis and early detection of disease, and accelerate drug development (Hanash 2003).

One may expect that with the new approach of drug research, including combinatorial chemistry, genomics, pharmacogenomics, proteomics, and bioinformatics, unprecedented results will be generated (Browne 2000; Burley et al. 1999; Debouck and Metcalf 2000; Drews 2000; Haystead 2001).

The difficulties arising in proteomic experiments from data analysis are discussed by Patterson (2003).

Duncan and Hunsucker (2005) discussed the value of proteomics as a tool for clinically relevant biomarker discovery and validation. A critical review on the impact of "OMIC" technology in drug development is given by Bilello (2005).

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0.4.6

Array Technology

Array technology is based on the RNA and DNA hybridization reaction previously used in Northern and Southern expression analysis. However, these traditional approaches allowed the detection of only one single gene. In contrast, gene arrays and microarrays allow the analysis of hundreds or thousands of genes simultaneously (DeRisi and Iyer 1999; Diehn et al. 2000; Epstein and Butow 2000; Deyholos et al. 2001; Jordan 2001; Rocket 2000; Taniguchi 2001; Hardiman 2004; Imbeaud and Auffray 2005). Gene chips can be global, containing the entire genome of one species on the slide. Besides the miniaturization, the greatest leap of technology was the development of fluorescence-labeled nucleotides, which can be detected by laser scanning. They have now replaced the use of radioactively labeled DNA.

Two different methods are used in array production: chips are loaded with either synthetic oligonucleotides (Lipshutz et al. 1999) or with a DNA fragment isolated directly from the respective gene. A specific process

allows the synthesis of oligonucleotides directly onto the glass surface. Normally, every gene is represented by about 20 oligonucleotides from different regions of the gene. Each oligonucleotide is 25–30 base pairs in length and is synthesized next to a control oligonucleotide with a mismatch. Whole genome arrays were pioneered by Affymetrix using a proprietary chip technology but are now available from other suppliers as well.

The development of DNA chips was pioneered by researchers at Stanford University (Brown and Botstein 1999; Sherlock et al. 2001). Along with the Stanford Microarray Database a number of additional centers voted for an open online access to their array databases: <http://genome-www.stanford.edu>.

Killion et al. (2003) created the Longhorn Array Database (LAD), which is an open-source, MIAME-compliant implementation of the Stanford Microarray Database. LAD is available at <http://www.longhornarraydatabase.org/>.

The standard MIAME (minimum information about a microarray experiment) was proposed by Brazma et al. (2001).

Kamb and Ramaswami (2001) presented a simple method for statistical analysis of intensity differences in microarray-derived gene expression data which does not require multiple replicates. The difference-averaging method enables determination of variances as a function of signal intensities by averaging over the entire dataset.

Novoradovskaya et al. (2004) recommended Universal Reference RNA (URR) as a standard for microarray experiments, a useful tool for monitoring intra- and inter-experimental variation. A variety of microassays were used to determine the percentage of spots hybridizing with URR and producing signal above a user defined threshold (microarray coverage). Microarray coverage was consistently greater than 80% for all arrays tested.

Regardless of using oligonucleotides or DNA arrays, one always needs two RNA samples for chip analysis. The RNA can be isolated from treated versus untreated cell lines, from the serum of an animal before or after drug administration, or from tumor versus normal tissue. Standard protocols require about 100 µg total RNA, from which poly A⁺ RNA is extracted. Next, the RNA is converted by reverse transcription into cDNA in the presence of fluorescence-labeled dyes. Commonly, the sample is labeled with Cy-5 and the control with Cy-3. Both cDNAs are combined and hybridized against the array (Rautenstrauss and Liehr 2001). Since DNA–DNA binding

reactions are thermodynamically slow, the chip is incubated overnight at temperatures above 60°C. Chip analysis is performed by scanning with two lasers at 562 nm (Cy-3) and 644 nm (Cy-5). The overlay of both scans shows genes that are deregulated. By convention, overexpressed genes are shown in green, repressed genes in red and unchanged expression in yellow. Data are evaluated by scatter blot analysis, where the relative expression of each gene is indicated by logarithmic axes. Sophisticated bioinformatic protocols are used for further annotation and data comparison (Zhang 1999; Gaasterland and Berikanow 2000).

The best approach to microarray analysis is to begin with a small number of the elements in the microarray known to be a pattern and ask questions of the other elements in the microarray; i. e., perform instantaneous scientific experiments regarding whether each of the other elements in the microarray is related to the known pattern (Burke 2000).

Emerging technologies and perspectives for microarray applications are reviewed by Gebauer (2004).

Epstein and Walt (2003) described fluorescence-based fibre optic arrays as a universal platform for sensing. Optical fibres provide a universal sensing platform as they are easily integrated with a multitude of different sensing schemes. Such schemes enable the preparation of a multitude of sensors including high-density oligonucleotide arrays and high-throughput cell-based arrays. Imaging fibre bundles comprised of thousands of fused optical fibres are the basis for an optically connected, individually addressable parallel sensing platform. Fibre optic imaging bundles possess miniature feature sizes (3- to 10- μm -diameter fibres), allowing high-density sensor packing ($\sim 2 \times 10^7$ sensors per cm^2).

Ge (2000) described the development of a universal protein array (UPA) system that provides a sensitive, quantitative, multipurpose, effective and easy technology to determine not only specific protein–protein interactions, but also specific interactions of proteins with DNA, RNA, ligands and other small chemicals.

The application of biochip and microarray systems in pharmacogenomics is discussed by Jain (2000).

MacBeath et al. (1999) discussed the possibilities of printing small molecules as microassays and detecting protein–ligand interactions en masse.

Firestein and Pisetsky (2002) published guidelines for studies using DNA microarray technology. The criteria for evaluating microarray studies are: (1) reproducibility must be demonstrated, including rigorous evaluation of the run-to-run variability of each gene; (2) detailed statistical analysis is required; including

appropriate corrections for repeated multiple measurements; (3) homogeneous cell populations should be optimally studied, to reduce the complexity of analysis; (4) a non-array method must confirm changes in the expression of key genes.

Ziauddin and Sabatini (2001) developed a microarray-driven gene expression system for the functional analysis of many gene products in parallel. Mammalian cells are cultured on a glass slide printed in defined locations with different DNAs. Cells growing on the printed areas take up the DNA, creating spots of localized transfection within a lawn of non-transfected cells. By printing sets of complementary DNAs cloned in expression vectors, microarrays are made, whose features are clusters of live cells that express a defined cDNA at each location.

Feldman et al. (2002) described advantages of mRNA amplification for microarray analysis and recommended routine mRNA amplification for all cDNA microarray-based analysis of gene expression.

Nielsen et al. (2005) developed a ready-to-spot polymer microarray slide, which is coated with a uniform layer of reactive electrophilic groups using anthraquinone-mediated photo-coupling chemistry. The slide coating reduces the hydrophobicity of the native polymer significantly, thereby enabling robust and efficient one-step coupling of spotted 5'-amino-like oligonucleotides onto the polymer slide. The coated polymer microarray slide represents a robust and cost-effective platform for pre-spotted oligonucleotide arrays.

Luo and Geschwind (2001) reviewed microarray applications in neuroscience with reference to applications in neurologic diseases in humans and the use of animal models.

Bunney et al. (2003) reviewed new strategies to discover candidate vulnerability genes in psychiatric disorders by microarray technology.

Templin et al. (2002) discussed the possibilities offered by protein microarray technology. Recent developments in the field of protein microarrays show applications for enzyme–substrate, DNA–protein and different types of protein–protein interactions. The authors discuss theoretical advantages and limitations of any miniaturized capture-molecule–ligand assay system.

Salisbury et al. (2002) described peptide microarrays for the determination of protease substrate specificity using fluorogenic peptidyl coumarin substrates.

The technology, the materials and the methods used for antibody microarrays are described by Lal et al. (2002) and Kusnezow et al. (2003).

Gopalakrishnan et al. (2002) described the application of Micro Arrayed Compound Screening (microARCS) to identify inhibitors of caspase-3. In this format, 8,640 discrete compounds are spotted and dried onto a polystyrene sheet, which has the same footprint as a 96-well plate. A homogeneous time-resolved fluorescence assay format (LANCE) was applied to identify the inhibitors of caspase-3 using a peptide substrate labeled with a fluorescent europium chelate and a dabcyI quencher. The caspase-3 enzyme was cast into a thin agarose gel, which was placed on a sheet containing test compounds. A second gel containing caspase substrate was then laid above the enzyme gel to initiate the reaction. Caspase-3 cleaves the substrate and separates the europium from the quencher, giving rise to a time-resolved fluorescence signal, which was detected using a ViewLux charge-coupled device imaging system.

Protein chip technology has been addressed by Zhu and Snyder (2003). Protein microarray technology has shown its great potential in basic research, diagnostics, and drug discovery. It has been applied to analyze antibody-antigen, protein-protein, protein-nucleic acid, protein-lipid and protein-small molecule interactions, as well as enzyme-substrate interactions. Progress in the field of protein chips includes surface chemistry, capture molecule attachment, protein labeling and detection methods, high-throughput protein-antibody production, and applications to analyze entire proteomes.

Lenigk et al. (2002) recommended plastic biochannel hybridization devices as a new concept for microfluidic DNA arrays.

Lee et al. (2004) described high-throughput screening of novel peptide inhibitors of an integrin receptor from the hexapeptide library by using a protein microarray chip. Novel peptide ligands with high affinity to the integrin were identified from the peptide libraries with this chip-based screening system by a competitive inhibition assay in a simultaneous and high-throughput fashion.

Hsieh et al. (2004) described an ultra-high-throughput microarray generation and liquid dispensing using multiple disposable piezoelectric ejectors.

Lim et al. (2005) used microarray analysis to show that some microRNAs downregulate a large number of target mRNAs.

Howbrook et al. (2003) reviewed developments in microarray technologies including gene expression analysis, DNA microarrays, protein and peptide microarrays, glycomics, tissue and cell microarrays as well as progress in microarray technology, such as

microfluids, molecular imprintation, and nanotechnology.

Gosalia and Diamond (2003) developed a method that performs enzymatic assays in nanoliter volumes in the liquid phase with minimal evaporation, no cross-contamination, and high reproducibility, and that has the capability to rapidly assemble multicomponent reactions with minimal usage in a microarray format. Chemical compounds within individual nanoliter droplets of glycerol are microarrayed onto glass slides at 400 spots/cm². Using aerosol deposition, subsequent reagents and water are metered into each reaction center to rapidly assemble diverse multicomponent reactions without cross-contamination or the need for surface linkage. This proteomics technique allows the kinetic profiling of protease mixtures, protease-substrate interactions, and high-throughput screening reactions.

Kusnezow and Hoheisel (2002) discussed promises and problems of antibody microarrays and compared this approach with the achievements of DNA microarrays.

Rondelez et al. (2005) developed microfabricated arrays of femtoliter chambers that allow single molecule enzymology. The microchip ensures that the chambers are uniform and precisely positioned.

Rougemont and Hingamp (2003) provided a method for automatically determining relevant gene clusters among the many genes monitored with microarrays.

Forster et al. (2004) described triple-target microarray experiments as a novel experimental strategy. There are two main dye-labeling strategies for microarray studies based on custom-spotted cDNA or oligonucleotide arrays: (1) dye-labeling of a single target sample with a particular dye, followed by subsequent hybridization to a single microarray slide, (2) dye-labeling of two different target samples with two different dyes, followed by subsequent co-hybridization to a single microarray slide. The two dyes most frequently used for either method are Cy3 and Cy5. The authors proposed and evaluated a novel experimental set-up utilizing three differently labeled targets co-hybridized to one microarray slide. In addition to Cy3 and CY5, Alexa 594 was incorporated as a third dye label.

Sharma et al. (2004) developed software called "ArrayD" that offers various alternative design solutions for a microassay given a set of user requirements.

Watson et al. (2004) published methods for high-throughput validation of amplified fragment pools of bacterial artificial chromosome (BAC) DNA for con-

structuring high-resolution CHG assays. The development of array based on comparative genomic hybridization (CGH) technology provides improved resolution for detection of alterations in genomic DNA copy number. In array CHG, generating spotting solution is a multi-step process where BAC clones are converted to replenishable PCR-amplified fragment pools (AFP) for use as spotting solution in a microarray format on glass substrate. Current methods for identifying initial BAC clones can be adapted to verify the identity of AFP spotting solutions used in printing assays. Of these methods, AFP sequencing is considered to be the most efficient for large-scale identification of spotting solution in a high-throughput manner.

Daly et al. (2005) presented a statistical method based on propagation of error to evaluate concentration estimation errors in an ELISA microarray process.

Do and Choi (2006) gave a review of normalization techniques of microarray data from single-labeled platforms such as the Affymetrix GeneChip array to dual-labeled platforms, e. g., spotted array, focusing on their principles and assumptions.

Ma and Horiuchi (2006) recommended chemical microarray as a new tool for drug screening and discovery in order to find new ways in HTS to profile the activity of large numbers of chemicals against hundreds of biological targets in a fast, low-cost fashion.

Uttamchandani et al. (2005) reviewed recent advances and applications of small molecule microarrays. Small molecule microarrays (SMM) are of value in high-throughput exploration, both in the identification of biologically significant natural and synthetic small molecules and in harnessing their vast potential towards medicinal and diagnostic applications.

Sobek et al. (2006) recommended microarray technology as a universal tool for high-throughput analysis of biological systems and reviewed the recent developments in surface chemistry and derivatization.

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0.4.7 NMR Technology

Magnetic resonance imaging (MRI) and spectroscopy (MRS) have become established technologies in modern biomedical research, providing relevant information on the various stages of the drug discovery and development process and are an important tool in drug discovery and metabolism (Hoult et al. 1974; Rudin et al. 1999, 2004a, 2004b, 2006; Rudin and Weissleder 2003; Beckmann et al. 2001, 2005, 2006; Beckmann and Rudin 2006). Several successful and potential applications of MRI and MRS in stroke, rheumatoid arthritis, osteoarthritis, respiratory diseases, oncology and cardiovascular diseases are described.

Strengths of MRI are: (1) *high soft tissue contrast*, which is governed by a multitude of parameters; (2) *noninvasiveness*, which is relevant when studying chronic diseases and allows for the translation of study protocols from animals to humans; and (3) *the high chemical specificity* of MRS, allowing the identification of individual analytes based on compound-specific resonance frequencies, an important prerequisite for the *in vivo* study of tissue metabolism. The main disadvantage of MRI/MRS is the *inherently low sensitivity* due to the low quantum energy involved as compared to optical spectroscopy, leading to a low degree of spin polarization. Only nuclei with high natural abundance and high intrinsic magnetic moment, such as protons, provide sufficient signal intensity for imaging applications. In addition, exogenous contrast

agents (CA, paramagnetic or superparamagnetic compounds) with different levels of specificity are often used to modulate local signal intensities. MRI is a multidimensional technique providing cross-sectional images as well as full three-dimensional (3D) data of body regions.

Bammer et al. (2005) gave a review on the foundations of advanced MRI. The mathematical and physical basics as well as the medical applications of MRI are described by Hornak (2007).

Using time domain NMR techniques, slice selection with shaped pulses, special encoding by pulsed field gradients, gradient echo detection and Fourier transform methods, highly resolved images of anatomy can be generated in almost real time. The most frequently used parameters are the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1-mm-thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T_2 images are sensitive to water and, because all pathological alterations in brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualizing the spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T_1 values, whereas relatively loose structures, such as gray matter or lesions, have higher T_1 values. To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected ($0.1\text{--}0.3\text{ mmol kg}^{-1}$) and, in areas of increased blood-brain barrier permeability, leaks into the brain parenchyma, causing local enhancement of the T_1 -weighted signal intensity. Srijkers et al. (2007) reviewed the current status of MRI contrast agents.

Detailed descriptions on the use of MRI are reported in Chap. A, Sects. 3.2.6, 3.2.7, 8.1.13 and Chap. E, Sects. 10.1, 10.2, 10.2.1, 10.2.2, 10.2.3, 10.3, 10.3.1, and 10.3.2.

Sillerud and Larson (2006) reviewed screening methods for drug discovery based on nuclear magnetic resonance (NMR), which were boosted by the introduction of sophisticated computational techniques for reducing the time for the acquisition of the primary NMR data for multidimensional studies.

Further non-invasive radiological techniques, such as **BOLD-MRI** (blood-oxygen-level-dependent MRI; Ogawa et al. 1992; Keogh et al. 2005; Burke and

Bührle 2006), positron emission tomography (**PET**; Fowler et al. 1999) and ^{31}P **MRS** (Braun et al. 1997), have been evaluated.

With **BOLD-MRI**, brain function has been successfully probed and has initiated the widely practiced field of functional MRI (**fMRI**) (Andersen et al. 2002; Schwarz et al. 2003). Mirsattari et al. (2005) described physiological monitoring of small animals during fMRI. Sauter et al. (2002) applied fMRI to study functional recovery following cytoprotective treatment in a rat model of human embolic stroke.

In addition, spectroscopic imaging with *in vivo* magnetic resonance ^1H -spectroscopy MRI, known as **MRSI**, enables the quantification of relative concentrations of specific metabolites, such as intramyocellular lipid content, at predetermined locations in organs (Kurhanewicz et al. 1996, 2000; Kuhlmann et al. 2003; Neumann-Haefelin et al. 2004; Schoelch et al. 2004).

Another important MRI technique is magnetization transfer ratio (**MTR**) imaging. The MTR of a given tissue is defined as the ratio of free protons to protons bound to tissue macromolecules (Deloire-Grassin et al. 2000; Smith et al. 2006).

Among other methodologies in functional imaging is the use of electron paramagnetic resonance (**EPR**), a technique almost identical in principle and practice to NMR (Eaton et al. 1991). EPR addresses unpaired spins, and in a given magnetic field the resonance frequency of an ensemble of electrons is 658 times higher than that of protons. There are two approaches of EPR imaging (**ERPI**), namely the continuous-wave (CW) modality (constant frequency with a field sweep) or the time-domain (termed Fourier transform, FT) modality (acquisition of an impulse response of the system at constant field). Devasahayam et al. (2004) described tailored sinc pulses for uniform excitation and artifact-free radiofrequency time-domain EPR imaging. A truncated sinc $[\sin(x)/x]$ pulse, tailored to compensate for the Q-profile (RF frequency response) of the resonator, was shown to yield images from phantom objects as well as *in vivo* images, with minimal distortion.

Another very closely related modality known by two alternate names, **PEDRI** (proton electron double resonance imaging; Lurie et al. 1988, 2005) and **OMRI** (Overhauser-enhanced magnetic resonance imaging; Golman et al. 1998, 2002), utilizes the Overhauser effect, by which protons are polarized in the presence of stable free radicals to provide highly enhanced MR images at very low fields. The principle behind OMRI is the saturation of free radical resonance by continuous (or pulsed) irradiation

at the electron resonance frequency, which leads to an Overhauser enhancement of the proton magnetization (also known as dynamic nuclear polarization, DNP) via mainly the time-dependent dipolar interactions between the unpaired electrons and the water protons. This may lead to a high enhancement factor (Ardenkjær-Larsen et al. 1998). This technique has been shown to produce useful functional proton images that can be used to perform non-invasive quantitative *in vivo* oximetry. The sensitivity of OMRI can be additionally improved by field cycling (Lurie et al. 1989; Subramanian et al. 2004).

Krishna et al. (2002) used OMRI for tumor oximetry and co-recording of tumor anatomy and tissue oxygen concentration.

Post (2003) used exchange-transferred nuclear Overhauser effect spectroscopy (**et-NOESY**) to probe the conformation of a ligand while bound to its macromolecular receptor.

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0.5 Errors in Screening Procedures

Any screening procedure has a characteristic error rate. This is inevitable because in high-throughput screening it is necessary to sacrifice some accuracy or precision to achieve the requisite speed. Thus when a large number of compounds is carried through a particular screen, some of the compounds will be classified incor-

rectly. A screen may be used in an absolute sense, so that compounds that pass a certain criterion are termed positives, whereas those that fail to meet the criterion are termed negatives. Compounds that pass, but should have failed, are false positives. In general, false positives are tolerable, if they are not too numerous, because they will be rectified later. Compounds that fail, but should have passed, are false negatives. False negatives are lost forever if the failure eliminates them from further testing.

All screening procedures are based on assumptions of analogy. They have different degrees of relevance or predictability. Studies in phase II clinical trials will predict with high probability the results in large clinical trials. But even here there is the possibility of false-positive or false-negative results. The relevance of a test is much less in early pharmacological tests, such as used in high-throughput screening. Generally, the relevance is inversely proportional to the simplicity of the test.

In any case, one is confronted with the problem of false-positive results (type I errors) and false-negative results (type II errors).

In each step two sources of error for **false-positive results** have to be taken in account:

1. a = error of the first type due to the model
2. α = error of the first type due to statistics

For definition: In the error of the first type a compound is considered to be active, but is actually ineffective. This type of error will be clarified during further development, after negative clinical trials at the latest.

However, there are also two sources of error for **false-negative results**:

1. b = error of the second type due to the model
2. β = error of the second type due to statistics

In the error of the second type a compound is considered to be ineffective, but is actually effective.

This type of error will never be clarified; an effective drug has just been missed. Perhaps another investigator will test this compound under different aspects.

The statistical errors derive from the fact that a pharmacological test is performed only several times or in a limited number of animals. One can specify the probability that a decision made is incorrect, i. e., a drug candidate is erroneously identified as effective when it is actually ineffective. Usually this risk is set to 5% ($P < 0.05$) and is called the statistical error of the first type or type I error. The error of the second type or type II error is connected to the type I error by statistical rules.

Usually, screening is performed sequentially. Tests in high-throughput screening are followed by tests in

isolated organs, then in small animals, and special tests in higher animals, until the compound is recommended for further development and for studies in human beings. From each step errors of type I, but also from type II, arise. As a consequence, many effective compounds will be lost.

There are two ways to circumvent this obstacle: (1) to increase the number of compounds entering the screening procedure dramatically, hope for a reasonable number of true positives, and accept a high rate of false-negative results (White 2000) as followed in the ultra high-throughput screening, or (2) to perform tests with high relevance, meaning tests with high predictive value in whole animals at an early stage (Vogel and Vanderbeeke 1990).

The literature on high-throughput screening includes some publications dealing with false-negative results (Jones and King 2003; Colland and Daviet 2004; Heller-Uszynska and Kilian 2004).

Zhang et al. (1999, 2000) studied the role of false-negative results in high-throughput screening procedures. They presented a statistical model system that predicts the reliability of hits from a primary test as affected by the error in the assay and the choice of the hit threshold. The hit confirmation rate, as well as false-positive (representing substances that initially fall above the hit limit but whose true activity is below the hit limit) and false-negative (representing substances that initially fall below the hit limit but whose true activity is in fact greater than the hit limit) rates have been analyzed by computational simulation. The Z-factor and the Z'-factor were introduced to characterize the reliability of high-throughput assays.

The problem of type II errors, i. e., false-negative results, also exists in many other physiological and pharmacological studies (Martorana et al. 1982; Barros et al. 1991; Sandkühler et al. 1991; Waldeck 1996; Williams et al. 1997). For example, Pollard and Howard (1986) re-investigated the staircase test,

a well-accepted primary screening method for anxiolytics, and found several false-negative results for clinically active anxiolytics.

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Chapter A

Cardiovascular Activity¹

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¹With contributions to this and the former editions by B.A. Schölkens, H. Gögelein, F.P. Huger, W. Linz, K.A. Rudolphi, K. Wirth.

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|---------------------------------------|--------|
| D(+)-sucrose | 250 mM |
| pH 7.4 | |
| preparation buffer B (=rinse buffer): | |
| Tris-HCl | 50 mM |
| MgCl ₂ × 6H ₂ O | 10 mM |
| pH 7.4 | |
| incubation buffer: | |
| Tris-HCl | 50 mM |
| MgCl ₂ × 6H ₂ O | 10 mM |
| ascorbic acid | 1.6 mM |
| catechol | 0.3 mM |
| pH 7.4 | |
| radioligand: | |
| ³ H-prazosin × HCl | |
| specific activity | |
| 0.37–1.11 TBq/mmol | |
| (10–30 Ci/mmol) (NEN) | |

Tissue Preparation

Male Sprague-Dawley rats (200–300 g) are sacrificed by decapitation and the dissected hearts are placed in ice-cold preparation buffer A. After removal of the atria, ventricles (approx. 30 g from 40 rats) are minced with a scalpel into 2–3 mm pieces.

Membrane Preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/20 ml preparation buffer A), the homogenate is filtered through gauze, and centrifuged at 2000 g (4°C) for 10 min. The pellets are discarded; the supernatant is collected, and centrifuged again at 40,000 g for 20 min. The resulting pellets are resuspended in approx. 300 ml preparation buffer B, homogenized by Ultra-Turrax and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in preparation buffer B, corresponding to 1 g ventricle wet weight/4 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at –77°C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment the required volume of the membrane suspension is slowly thawed and centrifuged at 40,000 g (4°C) for 20 min. The pellets are

resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of 1.0–1.5 mg/ml. After homogenization by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

For each concentration samples are prepared in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

total binding:

- 50 µl ³H-prazosin (12 concentrations, 5 × 10⁻¹¹–5 × 10⁻⁹ M)
- 50 µl incubation buffer

non-specific binding:

- 50 µl ³H-prazosin (4 concentrations, 5 × 10⁻¹¹–5 × 10⁻⁹ M)
- 50 µl phentolamine (10⁻⁵ M)

Competition Experiments

- 50 µl ³H-prazosin (1 constant concentration, 2–3 × 10⁻¹⁰ M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10⁻¹⁰–10⁻³ M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1.0–1.5 mg protein/ml). The samples are incubated for 30 min in a shaking bath at 25°C. The reaction is stopped by withdrawing the total incubation volume by rapid vacuum filtration over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^3H -prazosin versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D \text{ } ^3\text{H} \times \text{IC}_{50}}{K_D \text{ } ^3\text{H} + [^3\text{H}]}$$

IC_{50} = concentration of the test drug, which competes 50% of specifically bound ^3H -prazosin in the competition experiment

$[^3\text{H}]$ = concentration of ^3H -prazosin in the competition experiment.

$K_D \text{ } ^3\text{H}$ = dissociation constant of ^3H -prazosin, determined from the saturation experiment.

The K_i -value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug. The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

MODIFICATION OF THE METHOD

Binding of ^3H -WB 4101 to α_1 -adrenoregic receptors in brain is used to test hypotensive activity as a possible side effect of neuroleptic drugs. The test E.5.1.6 is described in the chapter on neuroleptic activity.

Couldwell et al. (1993) found that the rat prostate gland possesses a typical α_1 -adrenoceptor similar to that found in the vas deferens.

SUBTYPES OF THE α_1 -ADRENOCEPTOR

Several subtypes of the α_1 -adrenoceptor have been identified by pharmacological means (α_{1A} and α_{1B} , α_{1C} , α_{1D} ; α_{1H} , α_{1L} and α_{1N} adrenoceptors; Endoh et al. 1992; García-Sáinz et al. 1992, 1993; Ohmura et al. 1992; Regan and Cotecchia 1992; Satoh et al. 1992; Schwinn and Lomasney 1992; Veenstra et al. 1992; Aboud et al. 1993; Oshita et al. 1993; Vargas et al. 1993; Ruffolo et al. 1994; Minneman and Esbenshade 1994; Alexander et al. 2001) or by recombinant technology ($\alpha_{1a/d}$, α_{1b} , α_{1c} adrenoceptors). They correspond to the pharmacologically defined α_{1A} , α_{1B} , and α_{1D} adrenoceptors in native tissues (Bylund et al. 1994, 1998; Hieble et al. 1995; Graham et al. 1996; Hieble and Ruffolo 1996; Alexander et al. 2001; Hawrylyshyn et al. 2004; Waitling 2006).

Binding of the radioligand [^3H]-prazosin to the α_{1A} -adrenoceptor subtype can be measured in membranes prepared from male Wistar rat submaxillary glands (Michel et al. 1989).

Binding of the radioligand [^3H]-prazosin to the α_{1B} -adrenoceptor subtype can be measured in membranes prepared from male Wistar rat livers (Adolfo et al. 1989).

According to Eltze and Boer (1992), the adrenoceptor agonist SDZ NVI 085 discriminates between α_{1A} - and α_{1B} -adrenoceptor subtypes in vas deferens, kidney and aorta of the rat and may therefore be used as a tool either to detect (rat vas deferens or kidney) or exclude (rat aorta) the functional involvement of " α_{1A} -adrenoceptors in smooth muscle contraction."

Stam et al. (1998) found that (+)-cyclazosin, which behaves as a selective, high-affinity α_{1B} -adrenoceptor ligand in binding experiments, did not show the profile of a α_{1B} -adrenoceptor antagonist in functional tissues.

Decreased blood pressure response in mice deficient of the α_{1b} -adrenoregic receptor was found by Cavalli et al. (1997).

Kenny et al. (1995) used the contractile response of rat aorta to adrenaline after the application of various α_1 -adrenoceptor antagonists for characterization of a α_{1D} -adrenoceptor.

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A.1.1.2

α_2 -Adrenoreceptor Binding

PURPOSE AND RATIONALE

α_2 -adrenoceptors are widely distributed and are activated by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla or from some neurons in the CNS. The most extensively characterized action is the prejunctionally mediated inhibition of the release of neurotransmitters from many peripheral and central neurons. α_2 -adrenoceptors are also present at postjunctional sites, where they mediate actions such as smooth muscle contraction, platelet aggregation and inhibition of insulin secretion. Activation of postsynaptic α_2 -adrenoceptors in the brainstem results in an inhibition of sympathetic outflow in the periphery.

Clonidine is a centrally-acting antihypertensive agent, which lowers blood pressure mostly through reducing sympathetic tone by acting at the nucleus tractus solitarius in the brain stem (Kobinger and Walld 1967). Clonidine can, however, act at both peripheral and central α_2 -receptors. Peripherally administered clonidine causes a brief increase in blood pressure followed by a prolonged decrease (Rand and Wilson 1968). Functional studies (^3H -NE release) indicate a presynaptic mechanism for clonidine (Langer 1977,

1981; Starke 1977). However, lesioning studies fail to confirm a presynaptic location for clonidine receptors in either the CNS or periphery (U'Prichard et al. 1979; Bylund and Martinez 1981; U'Prichard et al. 1980). No change in clonidine receptor sites was seen after 6-hydroxydopamine lesions in cerebral cortex. This may be due to the fact that α_2 -receptors are both pre- and postsynaptic (Hieble et al. 1988).

Alpha-adrenergic agonists most potently displace ^3H -clonidine. Ergot compounds, dopamine agonists and mianserin are also fairly potent (U'Prichard et al. 1977). A survey on functions mediated by alpha-2 adrenergic receptors was given by Ruffolo et al. (1988) and on the role of neurotransmitters in the central regulation of the cardiovascular system by McCall (1990). Although clonidine relieves the autonomic symptoms of morphine withdrawal (Gold et al. 1978), there is no evidence for a direct α_2 /opiate-receptor interaction.

The purpose of this assay is to assess the interaction of hypotensive agents with central α_2 -receptors and determine possible clonidine-like mechanisms of action. Clonidine binding may also be relevant to the activity of other classes of drugs such as antidepressants that interact with α_2 -receptors.

PROCEDURE

Reagents

- Tris buffer pH 7.7
 - 57.2 g Tris HCl q.s. to 1 liter (0.5 M Tris buffer, pH 7.7)
16.2 g Tris base
 - make a 1:10 dilution in distilled H_2O (0.05 M Tris buffer, pH 7.7)
- Tris buffer containing physiological ions
 - Stock buffer

| | |
|-----------------|---------|
| NaCl | 7.014 g |
| KCl | 0.372 g |
| CaCl_2 | 0.222 g |
| MgCl_2 | 0.204 g |

q.s. to 100 ml in 0.5 M Tris buffer
 - Dilute 1:10 in distilled H_2O .
This yields 0.05 M Tris HCl, pH 7.7; containing NaCl (120 mM), KCl (5 mM), CaCl_2 (2 mM) and MgCl_2 (1 mM)
- [4- ^3H]-Clonidine hydrochloride (20–30 Ci/mmol) is obtained from New England Nuclear.
For IC_{50} determinations: ^3H -Clonidine is made up to a concentration of 120 nM and 50 μl are added to each tube (yielding a final concentration of 3 nM in the 2 ml volume assay).

- Clonidine-HCl is obtained from Boehringer Ingelheim.

A stock solution of 0.1 mM clonidine is made up to determine non-specific binding. This yields a final concentration of 1 μM in the assay (20 μl to 2 ml).

- Test compounds:

For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay and higher or lower concentrations can be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation and the cortical tissue is rapidly dissected. The tissue is homogenized in 50 volumes of 0.05 M Tris buffer pH 7.7 (buffer 1b) with the Brinkman Polytron, and centrifuged at 40,000g for 15 min. The supernatant is discarded and the final pellet rehomogenized in 50 volumes of buffer 2b. This tissue suspension is then stored on ice. The final tissue concentration is 10 mg/ml. Specific binding is 1% of the total added ligand and 80% of total bound ligand.

Assay

- | | |
|--------------------|--------------------------------------------------------------------------------------------------------------|
| 100 μl | 0.5 M Tris – physiological salts pH 7.7 (buffer 2a) |
| 830 μl | H_2O |
| 20 μl | Vehicle (for total binding) or 0.1 mM clonidine (for nonspecific binding) or appropriate drug concentration. |
| 50 μl | ^3H -clonidine stock solution |
| 1000 μl | tissue suspension. |

Tissue homogenates are incubated for 20 min at 25°C with 3 nM ^3H -clonidine and varying drug concentrations, and immediately filtered under reduced pressure on Whatman GF-B filters. The filters are washed with 3 five ml volumes of 0.05 M Tris buffer pH 7.7, and transferred to scintillation vials. Specific clonidine binding is defined as the difference between total bound radioactivity and that bound in the presence of 1 μM clonidine.

EVALUATION

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATIONS OF THE METHOD

Perry and U'Prichard (1981) described [³H]rauwolscine (α -yohimbine) as a specific radioligand for brain α_2 -adrenergic receptors.

Goldberg and Robertson (1983) reviewed yohimbine as a pharmacological probe for the study of the α_2 -adrenoreceptor.

Pimoule et al. (1983) characterized [³H]RX 781094 [(imidazolyl-2)-2benzodioxane-1,4] as a specific α_2 -adrenoceptor antagonist radioligand.

Murphy and Bylund (1988) characterized alpha-2 adrenergic receptors in the OK cell, an opossum kidney cell line.

Binding of the radioligand [³H]-rauwolscine to the α_{2A} -adrenoceptor subtype can be measured in membranes prepared from rabbit spleens (Michel et al. 1989).

Binding of the radioligand [³H]-yohimbine to the α_{2B} -adrenoceptor subtype can be measured in membranes prepared from male Wistar rat kidney cortices (Connaughton and Docherty 1989).

SUBTYPES OF THE α_2 -ADRENOCEPTOR

Using ³H-rauwolscine as ligand Broadhurst et al. (1988) studied the existence of two alpha₂-adrenoceptor subtypes.

Bylund et al. (1988) used [³H]-yohimbine and [³H]-rauwolscine to study alpha-2A and alpha-2B adrenergic subtypes in tissues and cell lines containing only one subtype.

Brown et al. (1990) found that [³H]-yohimbine labels at α_{2A} - and α_{2B} -adrenoceptors whereas [³H]-idazoxan labels the α_{2A} -adrenoceptor and, in addition, an imidazoline binding site.

Several subtypes of the α_2 -adrenoceptor have been identified by pharmacological means (α_{2A} -, α_{2B} -, α_{2C} -, and α_{2D} -adrenoceptors; Ruffolo 1990; Uhlén and Wikberg 1990; Gleason and Hieble 1992; Satoh and Takayanagi 1992; Takano et al. 1992; Ruffolo et al. 1993) or by recombinant technology as α_{2a} -, α_{2b} -, α_{2c} -adrenoceptors (Bylund et al. 1994; Hieble et al. 1995; Hieble and Ruffolo 1996; Alexander et al. 2001; Waitling 2006).

Gleason and Hieble (1992) reported that the α_2 -adrenoreceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the α_{2C} -adrenoceptor.

Marjamäki et al. (1993) recommended the use of recombinant human α_2 -adrenoceptors to characterize subtype selectivity of antagonist binding.

All three α_2 -adrenoceptor types serve as autoreceptors in postganglionic sympathetic neurons (Trendelenburg et al. 2003).

Uhlén et al. (1994) found that the α_2 -adrenergic radioligand [³H]-MK912 is α_{2C} -selective among human α_{2A} -, α_{2B} - and α_{2C} -adrenoceptors.

Uhlén et al. (1998) tested the binding of the radioligand [³H]RS79948-197 to human, guinea pig and pig α_{2A} -, α_{2B} - and α_{2C} -adrenoceptors and compared the values with MK912, RX821002, rauwolscine and yohimbine. [³H]RS79948-197 was non-selective for the α_2 -adrenoceptor subtypes, showing high affinity for all three.

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A.1.1.3

Electrically Stimulated Release of [3 H]Norepinephrine from Brain Slices

PURPOSE AND RATIONALE

The existence of presynaptic receptors which regulate the evoked release of neurotransmitters has been functionally demonstrated in both peripheral and central nervous system (Langer 1981; Starke 1981; Raiteri et al. 1984; Miller 1998). Presynaptic adrenergic α_2 -receptors regulate the evoked release of norepinephrine, comprising a short negative feedback loop. Alpha-2 agonists, such as clonidine and guanabenz, inhibit evoked release and alpha-2 antagonists, such as yohimbine and idazoxan, enhance evoked release.

The assay is used as a biochemical screen for agents which enhance or inhibit release of [3 H]norepinephrine (3 H-NE) and is particularly useful for testing receptor function of α_2 -adrenergic agonists and antagonists.

The procedures used emphasize delicate care of slices. By treating slices with great care, one is able to incubate at low tracer concentrations of 3 H-NE (25 nM), thus minimizing nonspecific labeling of releasable pools other than those in noradrenergic nerve terminals. It also permits the use of low (and more physiological) stimulation parameters, which allow the

neurons to recover easily between stimulations and do not flood the synaptic cleft with released NE, which would compete with any applied drug thus decreasing sensitivity.

PROCEDURE

This assay is based on the method described by Zahner et al. (1986).

A Reagents

1. Krebs-Henseleit bicarbonate buffer, pH 7.4 (KHBB):

| | |
|----------------------------------------|----------|
| NaCl | 118.4 mM |
| KCl | 4.7 mM |
| MgSO ₄ × 7 H ₂ O | 1.2 mM |
| KH ₂ PO ₄ | 2.2 mM |
| NaHCO ₃ | 24.9 mM |
| CaCl ₂ | 1.3 mM |
| dextrose (added prior to use) | 11.1 mM |

The buffer is aerated for 60 min with 95% O₂, 5% CO₂ on ice and pH is checked.

2. Levo-[Ring-2,5,6-³H]-norepinephrine (specific activity 40–50 Ci/mmol) is obtained from New England Nuclear.

The final desired concentration of ³H-NE is 25 nM. 0.125 nmol is added to 5 ml KHBB.

3. Test compounds

For most assays, a 1 mM stock solution of the test compound is made up in a suitable solvent and diluted such that the final concentration in the assay is 1 μM. Higher or lower concentrations may be used depending on the potency of the drug.

B Instrumentation

Neurotransmitter release apparatus consisting of:

- oscilloscope B8K, Precision Model 1420, dual-trace microscope (Dynascan Corp.)
- constant current unit, Grass model CCU1 (Grass Instr. Co.)
- stimulator, model S44, solid state square wave stimulator (Grass Instr. Co.)
- pump, Watson-Marlow, model 502 SHR, standard drive module; model 501 M multichannel pumphead (Bacon Technical Instr.)
- circulator, Haake D8 immersion circulator (Haake Buchler Instr. Inc.)
- fraction collector, Isco Retriever IV fraction collector (Isco Inc.)

C Tissue Preparation

Male Wistar rats (100–150 g) are decapitated, cortical tissue removed on ice and 0.4 mm slices are prepared with a McIlwain tissue chopper. The slices

are made individually and removed from the razor blade by twirling an artist's paint brush underneath the slice. Care should be taken not to compress the slice or impale it on the bristles. The slices are placed in cold, oxygenated buffer (10–20 ml) and incubated at 35°C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 5 ml of cold oxygenated buffer is added, and enough [³H]NE to bring the final concentration to 25 nM. This is then incubated and shaken for 30 min at 35°C under oxygen. After this step, the buffer is decanted and the "loaded" slices are rapidly placed on the nylon mesh in the stimulation chambers using a cut-off pipetman tip.

D Assay

To establish a stable baseline, control buffer is pumped through the chamber for 1 h at a flow rate of 0.7 ml/min before the first stimulation. One hour is allowed to pass before the second stimulation. When drugs are used, each concentration is prepared in a separate flask in control buffer and allowed to equilibrate with the tissue slice 20 min before the second stimulation. The experiment is stopped 40 min after the second stimulation.

Stimulation parameters are set at 5 Hz (2 ms duration) for 60 s, with 1 ms delay and voltage setting of 440 SIU (250 Ω).

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, then 200 ml of 20% methanol in distilled water, then distilled water again for at least 20 min.

EVALUATION

After conversion of dpm, percent fractional release is calculated for each fraction, using the spreadsheet program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue at that moment in time. "Spontaneous release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S₂/S₁ ratios. To normalize the data, drug effects can be estimated by first calculating S₂/S₁ values for control and drug-treated slices and then expressing the S₂/S₁ value for the drug-treated slices as a percentage of the

S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from each animal.

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A.1.1.4

Imidazoline Receptor Binding

PURPOSE AND RATIONALE

Imidazoline receptors constitute a family of nonadrenergic high-affinity binding sites for clonidine, idazoxan, and allied drugs. Drugs selectively binding to imidazoline receptors are expected to have less side effects than clonidine (Ernsberger et al. 1992, 1997; Molderings et al. 1992; Limon et al. 1992). One major subclass, the I_1 receptors, being mainly distributed in the brain and brain stem, partly mediates the central hypotensive action of clonidine-like drugs. The I_2 receptors, an other subclass, are mitochondrial, not G protein coupled, and have diversified functions. They may be involved in neuroprotection for cerebral ischemia. Two binding sites of [3 H]p-aminoclonidine, α_2 -adrenoceptors and imidazoline binding sites, could be separated (Ernsberger et al. 1987; Bricca et al. 1988; Kamisaki et al. 1990). At least 3 subtypes of imidazoline/guanidinium-receptive sites have been found by photoaffinity labeling (Lanier et al. 1993).

Several endogenous ligands for imidazoline receptors, collectively termed clonidine displacing substances (CDSs), have been detected in tissues and serum (Reis et al. 1995; Chan et al. 1997).

An endogenous substance with clonidine-like properties originally isolated from brain which binds selectively to imidazoline receptors was described by Atlas and Burstein (1984), Ernsberger et al. (1988), Atlas (1991), Meeley et al. (1992), Dontenwill et al. (1992, Ragunathan and Reis 1996). The endogenous substance agmatine, a decarboxylated arginine, may be the physiological agonist at imidazoline receptors acting as neurotransmitter (Li et al. 1994; Gonzales et al. 1996; Head et al. 1997; Herman 1997; Reis and Regu-

nathan 2000). Further candidates of endogenous ligands are discussed (Reis and Ragunathan 1998).

A critical review on imidazoline binding sites is given by Eglen et al. (1998).

PROCEDURE

Tissue Preparation

Whole bovine brains and adrenal glands are obtained from a local slaughterhouse. The lateral medulla oblongata is isolated by a sagittal section through the lateral margin of the pyramids and then bisected. The ventral half is defined as the ventrolateral medulla.

Fresh bovine adrenal glands are perfused retrogradely through the adrenal vein twice with 25 ml ice-cold Krebs-Henseleit bicarbonate buffer. The glands are perfused again with 25 ml ice-cold Krebs-Henseleit buffer containing 0.025% collagenase (type I, Sigma Chemical), incubated at room temperature for 1 h, then perfused with 25 ml fresh buffer containing collagenase and incubated for 30 min at 35°C. The digested glands are split, and the medulla is removed from the cortex. Adrenal medullae are minced and incubated while being stirred for 30 min at 37°C. The digest is filtered and centrifuged at 200 g for 30 min at 20°C. The cell pellet is resuspended in 30 ml Krebs' solution without collagenase, recentrifuged, flash-frozen, and stored at -70°C.

Membrane Preparation

Fresh bovine ventrolateral medulla and collagenase-digested rat renal medulla are homogenized with a Polytron (Tekmar Tissumizer; setting 80 for 15 s twice) in 20 vol of ice-chilled HEPES-buffered isotonic sucrose (pH 7.4) containing the protease inhibitors 1,10-phenanthroline (100 μ M) and phenylmethylsulfonyl fluoride (50 μ M). Bovine adrenomedullary chromaffin cells are homogenized in 15 ml HEPES-buffered isotonic sucrose by 10 strokes in a glass/glass hand-hold homogenizer. The homogenates are centrifuged at 1000 g for 5 min at 4°C to remove nuclei and debris. The pellets (P1) are resuspended in 20 ml of homogenization buffer and centrifuged again at 1000 g for 5 min. The supernatants are centrifuged at 48,000 g for 18 min at 4°C, and the resulting pellet (P2) is resuspended in 10–25 vol 50 mM Tris-HCl buffer (pH 7.7) containing 5 mM EDTA. After recentrifugation at 48,000 g for 18 min, the resulting membrane pellet is resuspended in Tris-HCl containing 25 mM NaCl, preincubated for 30 min at 25°C, chilled on ice, centrifuged again, resuspended a final time in Tris-HCl alone, centrifuged, flash-frozen, and stored at -70°C.

Binding Assays

For determination of specific binding to I₁-imidazoline sites and α_2 -adrenergic receptors radioligand binding assays are performed with [³H]clonidine, [³H]*p*-iodoclonidine, or [³H]moxonidine. Membranes are slowly thawed and resuspended in Tris-HCl or Tris-HEPES buffer (pH 7.7, 25°C). Assays are conducted in a total volume of 250 μ l in polypropylene 96 well plates (Beckman Macrowell). Each well contains 125 μ l membrane suspension, 25 μ l radioligand, and 100 μ l drug or vehicle. Incubations are initiated by the addition of membrane suspension and carried out for 40 min at 25°C. Nonspecific binding is defined in the presence of either piperoxan or phenotolamine (0.1 mM), which are imidazoline-adrenergic agents. Specific α_2 -adrenergic binding is defined by epinephrine (0.1 mM). In experiments with catecholamines, all samples contain ascorbic acid in a final concentration of 0.001%. Incubations are terminated by vacuum filtration over Reeves-Angel or Whatman GF/C fiberglass filters using a cell harvester (Brandel). The filters are washed four times with 5 ml ice-cold Tris-HCl, placed in scintillation vials, covered with 4 ml scintillation cocktail and counted at 50% efficiency. Protein is assayed by a modified Lowry et al. method (Peterson 1977) using a deoxycholate-trichloroacetic acid protein precipitation technique which provides a rapid quantitative recovery of soluble and membrane proteins from interfering substances even in very dilute solutions. Sodium dodecyl sulfate is added to alleviate possible nonionic and cationic detergent and lipid interferences, and to provide mild conditions for rapid denaturation of membrane and proteolipid proteins.

EVALUATION

Data are obtained as disintegrations per min and transferred to the Equilibrium Binding Data Analysis program (McPherson 1985). Then, several experiments are analyzed simultaneously with the LIGAND program for non-linear curve fitting (Munson and Rodbard 1980). IC₅₀ values are estimated from inhibition curves by non-linear curve fitting (Mutolsky and Ransnas 1987). Protein assay data are also analyzed by non-linear curve fitting (McPherson 1985).

MODIFICATIONS OF THE METHOD

Tesson et al. (1991) defined the subcellular localization of imidazoline-guanidinium-receptive sites by performing binding studies with the radioligand [³H]idazoxan.

Lanier et al. (1993) visualized multiple imidazoline/guanidinium-receptive sites with the photoaffinity adduct 2-[3-azido-4-[¹²⁵I]iodo-phenoxy]methyl imidazoline.

Molderings et al. (1991) characterized imidazoline receptors involved in the modulation of noradrenaline release in the rabbit pulmonary artery pre-incubated with [³H]noradrenaline.

Molderings and Göthert (1995) determined electrically or K⁺-evoked tritium overflow from superfused rabbit aortic strips pre-incubated with [³H]noradrenaline in order to characterize presynaptic imidazoline receptors which mediate noradrenaline release and compared them with I₁- and I₂-imidazoline radioligand binding sites.

Ernsberger et al. (1995) described optimization of radioligand binding assays for I₁ imidazoline sites.

Munk et al. (1996) reported the synthesis and pharmacological evaluation of a potent imidazoline-1 receptor specific agent.

Piletz et al. (1996) compared the affinities of several ligands for [¹²⁵I]*p*-iodoclonidine binding at human platelet I₁ imidazole binding sites.

Several selective ligands for imidazoline I₂ receptors have been identified, such as:

- LSL 60101 (Alemany et al. 1995; Menargues et al. 1995),
- RS-45041-190 (MacKinnon et al. 1995; Brown et al. 1995),
- RX801077 (= 2-BFI = 2-(2-benzofuranoyl)-2-imidazoline and analogues (Jordan et al. 1996; Lione et al. 1996; Alemany et al. 1997; Hosseini et al. 1997; Wiest and Steinberg 1997; Hudson et al. 1997).

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pancreas, stimulation of glycogenolysis in liver and skeletal muscle and stimulation of lipolysis in the adipocyte.

Three β -adrenoceptor proteins have been cloned, and the characteristics of these recombinant receptors correspond with those of the three well characterized β -adrenoceptors on native tissue, designated as β_1 , β_2 and β_3 . The possible roles of β_3 -adrenoceptors in the cardiovascular system were discussed by Gauthier et al. (2000). An additional β -adrenoceptor modulating cardiac contractility has been designated as the β_4 -adrenoceptor (Kaumann et al. 1998).

While it was initially thought that cardiac stimulation involved primarily the β_1 -adrenoceptor, it now appears that all of the receptor subtypes may be involved. Bronchodilation appears to be mediated by the β_2 -adrenoceptor. The β_3 -adrenoceptor is responsible for lipolysis in white adipose tissue and thermogenesis in the brown adipose tissue found in rodents. Renin release appears to be mediated by the β_1 -adrenoceptor (Waitling 2006).

The β -adrenoceptor population of plasma membranes from bovine heart ventricles consists of 75–80% β_1 - and 20–25% β_2 -adrenoceptors. The use of this tissue allows a parallel investigation of the binding characteristics of drugs at both the β_1 - and β_2 -adrenoceptors. Both, the β_1 - and β_2 -adrenoceptors coexist in rat ventricular myocytes, but stimulation of these receptor subtypes elicits qualitatively different cell responses at the levels of ionic channels, the myofilaments, and sarcoplasmic reticulum (Xiao and Lakatta 1993).

A constant concentration of the radioligand ³H-dihydroalprenolol (³H-DHA) (4–6 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine heart ventricles. If the test drug exhibits any affinity to β -adrenoceptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more effective is the test drug.

A.1.1.5

β -Adrenoceptor Binding

PURPOSE AND RATIONALE

β -adrenoceptors are widely distributed, found at both central and peripheral sites, and are activated either via norepinephrine released from sympathetic nerve terminals or via epinephrine released from the adrenal medulla. Important physiological consequences of β -adrenoceptor activation include stimulation of cardiac rate and force, relaxation of vascular, urogenital and bronchial smooth muscle, stimulation of renin secretion from the juxtaglomerular apparatus, stimulation of insulin and glucagon secretion from the endocrine

PROCEDURE

Materials and Solutions

preparation buffer:

| | |
|----------------------------------------|--------|
| Tris-HCl | 5 mM |
| MgCl ₂ × 6 H ₂ O | 1 mM |
| D(+)-sucrose | 250 mM |
| pH 7.4 | |

310 mOsm sodium phosphate buffer:

| | |
|----------------------------------------|---------------|
| pH 7.4 | rinse buffer: |
| Tris-HCl | 50 mM |
| MgCl ₂ × 6 H ₂ O | 10 mM |
| pH 7.4 | |

incubation buffer:

| | |
|----------------------------------------|--------|
| Tris-HCl | 50 mM |
| MgCl ₂ × 6 H ₂ O | 10 mM |
| ascorbic acid | 1.6 mM |
| catechol | 0.3 mM |
| pH 7.4 | |

radioligand:

(-)³H-dihydroalprenolol × HCl
³H-DHA specific activity 1.48–2.59 TBq/mmol
 (40–70 Ci/mmol) (NEN)

for inhibition of ³H-dihydroalprenolol binding in non-specific binding experiments:

(-)-isoprenaline(+)bitartrate salt (Sigma)

Bovine hearts are obtained freshly from the local slaughter house. The lower part of the left ventricle from 5 hearts is separated and kept in ice-cold preparation buffer. In the laboratory, approx. 60 g wet weight from the five ventricle pieces are minced with a scalpel into 2–3 mm pieces.

Membrane Preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/10 ml buffer), the homogenate is filtered through gauze and centrifuged at 500 g (4°C) for 10 min. The pellets are discarded, the supernatant is collected, and centrifuged at 40,000 g for 20 min. The resulting pellets are resuspended in approx. 300 ml 310 mOsm sodium phosphate buffer, homogenized by Ultra-Turrax, and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in sodium phosphate buffer corresponding to 1 g ventricle wet weight/2 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at -77°C. Protein concentration of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 40,000 g (4°C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of approx. 2.0 mg/ml. After homogenizing by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

All incubation samples are performed in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

total binding:

- 50 µl ³H-DHA
(12 concentrations, 3 × 10⁻¹⁰–4 × 10⁻⁸ M)
- 50 µl incubation buffer

non-specific binding:

- 50 µl ³H-DHA
(4 concentrations, 3 × 10⁻¹⁰–4 × 10⁻⁸ M)
- 50 µl (-)isoprenaline (10⁻⁵ M)

Competition Experiments

- 50 µl ³H-DHA
(1 constant concentration, 4–6 × 10⁻⁹ M)
- 50 µl incubation buffer without or with non-labeled test drug
(15 concentrations 10⁻¹⁰–10⁻³ M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (approx. 2 mg protein/ml). The samples are incubated for 60 min in a shaking water bath at 25°C. The reaction is stopped by rapid vacuum filtration of the total incubation volume over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ³H-DHA versus non-labeled drug by a computer-sup-

ported analysis of the binding data.

$$K_i = \frac{K_D \text{ } ^3\text{H} \times IC_{50}}{K_D \text{ } ^3\text{H} + [^3\text{H}]}$$

IC_{50} = concentration of the test drug, which competes with 50% of specifically bound ^3H -DHA in the competition experiment

$[^3\text{H}]$ = concentration of ^3H -DHA in the competition experiment.

$K_D \text{ } ^3\text{H}$ = dissociation constant of ^3H -DHA, determined from the saturation experiment.

The K_i -value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data

propranolol hydrochloride $K_i = 6-8 \times 10^{-9}$ mol/l

MODIFICATIONS OF THE METHOD

Abrahamsson et al. (1988) performed a receptor binding study on the β_1 - and β_2 -adrenoceptor affinity of atenolol and metoprolol in tissues from the rat, the guinea pig and man with various radioligands, such as [^{125}I](\pm)hydroxybenzylpindolol, [^{125}I]($-$)pindolol, [^3H]($-$)dihydroalprenolol, and [^3H]($-$)CGP 12177.

Fleisher and Pinna (1985) used specific binding of ($-$)[^3H]dihydroalprenolol to rat lung membranes for *in vitro* studies on the relative potency of bronchodilator agents.

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A.1.1.6

β_1 -Adrenoreceptor Binding

PURPOSE AND RATIONALE

β -adrenergic receptors were differentiated from α -receptors (Ahlquist 1948) and subsequently divided into 2 distinct subtypes, β_1 and β_2 (Lands et al. 1967) based on differing pharmacology in different tissues. β -receptors have been labelled in a number of tissues including heart, lung, erythrocytes and brain using the β -agonists [^3H]-epinephrine (U'Prichard et al. 1978), or [^3H]-hydroxybenzylisoproterenol (Lefkowitz and Williams 1977) or the β -receptor antagonists [^3H]-alprenolol (Mukherjee et al. 1975), [^3H]-dihydroalprenolol (DHA) (U'Prichard et al. 1978; Bylund and Snyder 1976) and (^{125}I)-iodohydroxypindolol (Weiland et al. 1980). DHA is a potent β -antagonist (Mukherjee et al. 1975), which labels both β_1 and β_2 adrenergic receptors. The binding characteristics of this ligand in brain were described by Bylund and Snyder (1976), who showed that antagonists competed potently and agonists less potently although stereospecificity was maintained. The pharmacology of binding was consistent with β_1 -receptor occupancy. Lesioning studies (Wolfe et al. 1982), combined with non-linear regression analysis of data have shown that while β -receptors in rat cerebellum are primarily of the β_2 subtype, the β_1 occurring in rat cerebral cortex are physiologically more significant. The assay can be used to evaluate the direct interaction of drugs with β -receptors labelled by [^3H]-dihydroalprenolol.

PROCEDURE

Reagents

Tris buffer, pH 8.0

1. a) 44.4 g Tris HCl q.s. to 1 liter
(0.5 M Tris, pH 8.0) 26.5 g Tris base
- b) Dilute 1:10 in distilled water.
(0.05 M Tris, pH 8.0)

2. (-)-[propyl-1,2,3-³H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.

For IC_{50} determinations: A stock solution of 20 nM ³H-DHA is made up in distilled H₂O and 50 μ l is added to each tube (this yields a final concentration of 1 nM in the 1 ml assay).

3. (\pm)-propranolol HCl is obtained from Ayerst.

A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 μ M propranolol solution. Twenty μ l of dilute stock solution is added to 3 tubes to determine nonspecific binding (yields a final concentration of 1 μ M in a 1 ml assay).

4. Test compounds:

For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound.

Tissue Preparation

Male rats are decapitated and the brains rapidly removed. The cerebral cortices are dissected free, weighed and homogenized in 50 ml of ice-cold 0.05 Tris buffer, pH 8.0. This homogenate is centrifuged at 40,000 *g*, the supernatant decanted and the pellet resuspended and recentrifuged at 40,000 *g*. The final pellet is resuspended in the initial volume of fresh 0.05 Tris buffer, pH 8.0. This tissue suspension is then stored on ice. The final tissue concentration in the assay is 10 mg/ml. Specific binding is about 3% of the total added ligand and 80% of the total bound ligand.

Assay

| | |
|-------------|-----------------------------------------------------------------------------------------------------------------------------|
| 380 μ l | H ₂ O |
| 50 μ l | 0.5 Tris buffer, pH 8.0 |
| 20 μ l | Vehicle (for total binding) or 50 μ M (\pm) propranolol (for nonspecific binding) or appropriate drug concentration |
| 50 μ l | ³ H-DHA stock solution |
| 500 μ l | tissue suspension. |

The tissue homogenates are incubated for 15 min at 25°C with 1 nM ³H-DHA and varying drug concentrations. With each binding assay, triplicate samples are incubated with 1 μ M (\pm)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice-

cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dooley et al. (1986) recommended CGP 20712 A as a useful tool for quantitating β_1 and β_2 adrenoceptors.

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A.1.1.7

β_2 -Adrenoreceptor Binding

PURPOSE AND RATIONALE

Lands et al. (1967) classified β -receptors into β_1 and β_2 subtypes according to differences in the action of various catecholamines. Synthesis of more selective β -antagonists has helped to confirm the existence of receptor subtypes. Based on catecholamine pharmacology and differences in the tissue distribution, it has been suggested that the β_1 -receptor serves as the receptor for norepinephrine acting as a neurotransmitter and the β_2 -receptor serves as a receptor for epinephrine acting as a hormone. (Nahorski 1981;

Ariens and Simonis 1983; Lefkowitz et al. 1983; Minneman 1983). Since [^3H]-dihydroalprenolol is a non-specific ligand, it is necessary to select a tissue which is enriched in β_2 -receptors in order to convey specificity to this assay. Tissues with predominantly β_2 -receptors include lung (U'Prichard et al. 1978; Ariens and Simonis 1983; Lefkowitz et al. 1983), cerebellum (Lefkowitz et al. 1983; Minneman et al. 1983), rat and frog erythrocytes (Mukherjee et al. 1975; Lefkowitz et al. 1983) and ciliary process (Nathanson 1985) whereas, forebrain, heart and avian erythrocytes are relatively enriched in the β_1 -subtype (Lefkowitz et al. 1983). Due to poor binding characteristics in cerebellum, rat lung is chosen as the tissue for β_2 -adrenergic receptors.

A compound with β_2 -selectivity would be less likely to produce cardiac effects but more likely to produce bronchiolar constriction. The test is used to determine the affinity of compounds for the β_2 -adrenergic receptor subtype. A measure of receptor subtype selectivity can be determined when data are compared with those obtained in the β_1 -adrenergic assay in rat cerebral cortex.

The present nomenclature of β_1 , β_2 , and β_3 receptors was reviewed by Alexander et al. (2001).

PROCEDURE

Reagents

- Tris buffers, pH 8.0
 - 44.4 g Tris HCl q.s. to 1 liter (0.5 M Tris, pH 8.0) 26.5 g Tris base
 - Dilute 1:10 in distilled water (0.05 M Tris, pH 8.0)
- (-)-[propyl-1,2,3- ^3H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
For IC_{50} determinations: A stock solution of 20 nM ^3H -DHA is made up in distilled water and 50 μl is added to each tube (this yields a final concentration of 1 nM in the assay)
- (\pm)-propranolol HCl is obtained from Ayerst.
A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 μM propranolol solution. Twenty μl of dilute stock solution are added to 3 tubes to determine nonspecific binding (yielding a final concentration of 1 μM in a 1 ml assay).
- Test compounds:
For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for

each assay. Higher or lower concentrations may be used depending on the potency of the compound to be tested.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation and the lungs removed, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 8.0 using a Tekmar homogenizer. The homogenate is passed through a cheese cloth and centrifuged at 40,000 g for 15 min. The final membrane pellet is resuspended in the original volume of Tris buffer, pH 8.0, and used in the assay.

Assay

| | |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------|
| 380 μl | H ₂ O |
| 50 μl | 0.5 Tris buffer, pH 8.0 |
| 20 μl | Vehicle (for total binding) or 50 μM (\pm)-propranolol (for nonspecific binding) or appropriate drug concentration |
| 50 μl | ^3H -DHA stock solution |
| 500 μl | tissue suspension. |

The tissue homogenates are incubated for 15 min at 25°C with 1 nM ^3H -DHA and varying drug concentrations. In each binding assay, triplicate samples are incubated with 1 μM (\pm)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice-cold 0.05 M Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dooley et al. (1986) recommended CGP 20712 as a useful tool for quantitating β_1 - and β_2 -adrenoceptors.

McCrea and Hill (1993) described salmeterol as a long-acting β -adrenoceptor agonist mediating cyclic AMP accumulation in the B50 neuroblastoma cell line.

Sarsero et al. (1998) recommended (-)[^3H]-CGP 12177A as radioligand for the putative β_4 -adrenoceptor.

McConnell et al. (1991, 1992; Owicki and Parce 1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells

were transfected with human β_2 -adrenergic receptors. The β_2 -adrenergic receptor activates adenylyl cyclase resulting in an increase in the cyclic AMP concentration within the cell which can be measured as acidification. Addition of 10 μ M isoproterenol, 500 μ M 8-bromo cyclic AMP, or 10 μ g/ml forskolin induced a reversible acidification.

Hoffmann et al. (2004) compared human β -adrenergic receptor subtypes using characterization of stably transfected receptors in CHO cells.

PROCEDURE

cDNA of human β -adrenergic receptors cDNAs coding for human β -adrenergic receptors in pcDNA3 expression vectors were verified by sequencing and comparison with the respective GeneBank entries. The translated amino acid sequences corresponded to the published sequences for the β_1 -adrenergic receptor (Frielle et al. 1987), β_2 -adrenergic receptor (Schofield et al. 1987), and β_3 -adrenergic receptor (Emorine et al. 1989). With respect to polymorphisms, the β -adrenergic receptors used in this study corresponded to the following variants: β_1 -receptor 49-Ser, 389-Gly; β_2 -receptor 16-Arg, 27-Gln, 164-Thr; β_3 -receptor 64-Trp. All of the variants correspond to the sequences originally termed wild-type.

Stable Transfection of Cells

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, Md., USA) were transfected with plasmid DNA for stable expression using the calcium phosphate precipitation method (Chen and Okayama 1987) as described for the rat A_1 adenosine receptor (Freund et al. 1994). Positive clones were selected with 600 μ g/ml of the neomycin analog G-418, and single clonal lines were isolated by limiting dilution. Expression of the receptor was verified by radioligand binding.

Cell culture and membrane preparation Chinese hamster ovary cells stably transfected with human β -adrenergic receptor subtypes were grown adherently and maintained in Dulbecco's Modified Eagle's Medium with nutrient mixture F12 (DMEM/F12), containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM) and geneticin (G-418, 0.2 mg/ml) at 37°C in 5% CO₂/95% air. Cells were split 2 or 3 times weekly at a ratio of between 1:5 and 1:15. In order to harvest cells the culture medium was removed, cells were washed twice with PBS and membranes were prepared or cells were frozen on the dishes for later preparation of membranes. Crude membrane fractions were prepared

from fresh (measurement of adenylyl cyclase) or frozen cells (radioligand binding). The resulting membrane pellets were resuspended in 50 mM Tris/HCl buffer pH 7.4 to give a final protein concentration of 1–2 mg/ml.

Radioligand Binding Studies and Adenylyl Cyclase Activity

The radioligand binding experiments were performed with membranes prepared as described above. Assays were done in a volume of 200 μ l in 50 mM Tris/HCl, pH 7.4 (assay buffer) in the presence of 100 μ M GTP to ensure monophasic binding curves for agonists. For saturation binding experiments at human β_1 - and β_2 -receptors up to 400 pM ¹²⁵I-CYP and for β_3 -receptors up to 1,500 pM ¹²⁵I-CYP were used. Non-specific binding was determined in the presence of 10 μ M alprenolol. For competition binding, 50 pM ¹²⁵I-CYP in the case of β_1 - and β_2 -receptors, or 80 pM ¹²⁵I-CYP for β_3 -receptors were used. For most of the competition binding experiments membranes with intermediate receptor expression (β_1 : 367 \pm 75 fmol/mg protein, β_2 : 282 \pm 19 fmol/mg protein, β_3 : 377 \pm 82 fmol/mg protein) were used. For selected compounds it was demonstrated that higher receptor expression did not affect K_i values (data not shown). Membranes were incubated for 90 min at 30°C, filtered through Whatman GF/C filters, and washed 3 times with ice-cold assay buffer. Samples were counted in a γ -counter (Wallac 1480 wizard 3). K_D -values for ¹²⁵I-CYP were calculated by non-linear curve fitting with the program SCT-FIT. Ligand IC₅₀ values were calculated using Origin 6.1 (OriginLab Corporation, Northampton, Mass., USA) and were transformed to K_i values according to Cheng and Prusoff (1973).

Adenylyl cyclase activity in cell membranes was determined according to Jakobs et al. (1976). Membrane protein (50 μ g) was added to an incubation mixture with final concentrations of 50 mM Tris/HCl pH 7.4, 100 μ M cAMP, 0.2% BSA, 10 μ M GTP, 100 μ M ATP, 1 mM MgCl₂, 100 μ M IBMX, 15 mM phosphocreatine, and 300 U/ml of creatine kinase. Membranes were incubated with about 200,000 cpm of [α -³²P]-ATP for 20 min in the incubation mixture as described (Klotz et al. 1985). Accumulation of [α -³²P]-cAMP was linear over at least 20 min under all conditions. The reaction was stopped by addition of 400 μ l of 125 mM ZnAc-solution and 500 μ l of 144 mM Na₂CO₃. Samples were centrifuged for 5 min at 14,000 rpm in a laboratory microcentrifuge. Then, 800 μ l of the resulting supernatant was finally applied to alumina WN-6 (Sigma) columns that were eluted twice with 2 ml of 100 mM Tris/HCl pH 7.4.

The eluates were counted in a β -counter (Beckmann LS 1801).

Niclauss et al. (2006) compared the ability of three radioligands, [125 I]-cyanopindolol, [3 H]-CGP 12,177 and [3 H]-dihydroalprenolol, to label the three human β -adrenoceptor subtypes. Saturation and competition binding experiments were performed using membrane preparations from Chinese hamster ovary cells stably transfected with the three subtypes. While [3 H]-CGP 12,177 had very similar affinity for β_1 - and β_2 -adrenoceptors (about 40 pM), [125 I]-cyanopindolol and [3 H]-dihydroalprenolol had four- to sixfold higher affinity for β_2 - as compared to β_1 -adrenoceptors (10 vs. 45 and 187 vs. 1,021 pM, respectively). The affinity of [125 I]-cyanopindolol at β_3 -adrenoceptors was considerably lower (440 pM) than at the other two subtypes. The β_3 -adrenoceptor affinity of [3 H]-CGP 12,177 and [3 H]-dihydroalprenolol was so low that it could not be estimated within the tested range of radioligand concentrations (up to 4,000 pM and 30,000 pM for [3 H]-CGP 12,177 and [3 H]-dihydroalprenolol, respectively). All three radioligands were ill-suited to labeling β_3 -adrenoceptors, particularly in preparations co-expressing multiple subtypes. In the absence of alternatives, [125 I]-cyanopindolol appears the least unsuitable for labeling β_3 -adrenoceptors. At present, there is still a need for high-affinity radioligands that are selective for β_3 -adrenoceptors.

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A.1.1.8

Adenosine A₁ Receptor Binding

GENERAL CONSIDERATIONS

Adenosine receptors belong to the class of purinoceptors (Burnstock 1972, 1981; Olsson and Pearson 1990). Purinoceptors are divided into two general types on the basis of recognized natural ligands:

P₁ receptors recognize adenosine and AMP and P₂ receptors recognize ATP and AMP. Fredholm et al. (1994), Abbracchio and Burnstock (1994, Jacobson et al. 2000) proposed a nomenclature system which is now widely accepted: two families of P₂ purinoceptors, P_{2X} ionotropic ligand-gated ion channel receptors (North 2002) and P_{2Y} metabotropic G-protein-coupled receptors (Costanzi et al. 2004). The nomenclature of seven subtypes of P_{2X} receptors and six subtypes of P_{2Y} receptors has been agreed by the NC-IUPHAR Subcommittee (Burnstock 2001; Alexander et al. 2001; Waitling 2006).

The effects of adenosine are mediated effects through cAMP (Sattin and Rall 1970; VanCalker et al. 1978). It was discovered that adenosine could either inhibit or stimulate the formation of cAMP. The discovery of dual effects on adenylate cyclase led to the proposal of two distinct adenosine receptors referred to as the A₁ and A₂ receptors. The A₁ subtype of adenosine receptor mediates the inhibition of adenylate cyclase; whereas, the A₂ subtype mediates stimulation of adenylate cyclase. The methylxanthines are relatively nonselective inhibitors of adenosine receptor subtypes and their pharmacological properties are thought to be mostly due to antagonism of these receptors.

Comparison of adenosine receptors with other G-protein linked receptors indicates that they comprise a family of G protein coupled receptors that can be grouped by subtypes or by species. Thus, in addition to A₁ and A₂, several authors described A_{1a}, A_{1b}, A_{2a}, A_{2b}, A₃, and A₄ receptors with species dependent differences (Jacobson et al. 1992 1996; Zhou et al. 1992; Linden et al. 1993; Salvatore et al. 1993; Linden et al. 1994; Fredholm et al. 1994; Alexander et al. 2001). The nomenclature and classification of adenosine receptors were published by Fredholm et al. (2001) and Waitling (2006). Klotz (2000) reviewed adenosine receptors and their ligands. The four subtypes of adenosine receptors referred to as A₁, A_{2A}, A_{2B}, and A₃ are members of the superfamily of G-

protein-coupled receptors. The most recently discovered member of the adenosine receptor family, the A₃ receptor, has a unique pharmacological profile (Salvatore et al. 1993; Avila et al. 2002).

PURPOSE AND RATIONALE

The purpose of this assay is to measure the affinity of test compounds for adenosine (A₁) receptors. Evidence for an A₁ adenosine receptor in the guinea pig atrium was given by Collis (1983). Adenosine plays a physiological role in many systems, including platelet aggregation, lipolysis, steroidogenesis and smooth muscle tone (Daly 1982). The vasodilatory and cardiac depressant effects of adenosine are well known. In addition to cardiovascular effects, adenosine has marked effects in the CNS including depression of electrophysiological activity (Siggins and Schubert 1981), anticonvulsant activity, analgesic properties (Ahlijanian and Takemori 1985) and inhibition of neurotransmitter release (Harms et al. 1979).

The agonist, [3 H]cyclohexyladenosine (CHA), has affinity for the A₁ receptor in the nanomolar concentration range and has proven to be a suitable ligand for A₁ receptor assays (Bruns et al. 1980; Bruns et al. 1986). Selective A₁ (Schingnitz et al. 1991) and A₂ antagonists (Shimada et al. 1992; Jacobson et al. 1993) have been described. Adenosine and its nucleotides have not only a cardiovascular but predominantly a cerebral activity (Phillis and Wu 1981; Daly 1982; Fredholm et al. 1982).

PROCEDURE

Reagents

1. a) 0.5 M Tris buffer, pH 7.7
b) 0.05 M Tris buffer, pH 7.7
2. Adenosine deaminase is obtained from Sigma Chemical Co.
Adenosine deaminase is added to 0.05 M Tris-HCl buffer, pH 7.7 for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.
3. Cyclohexyladenosine, N⁶-[Adenine-2,8- 3 H] (specific activity 34 mCi/mmol) is obtained from New England Nuclear.
For IC₅₀ determinations: [3 H]CHA is made up to a concentration of 40 nM and 50 μ l are added to each tube. This yields a final concentration of 1 nM in the assay.
4. Theophylline is obtained from Regis Chemical Co. A 100 mM stock solution is made up in deionized water. 20 μ l are added to each of 3 tubes for the de-

termination of nonspecific binding, yielding a 1 mM final concentration in the assay.

5. Test compounds

For most assays, a 1 mM stock solution is prepared in DMSO and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebellum are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 g for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellet is resuspended in 0.05 M Tris buffer containing 0.1 U/ml of adenosine deaminase.

Assay

| | |
|--------------|-----------------------------------------------------------------------------|
| 1000 μ l | tissue suspension |
| 930 μ l | H ₂ O |
| 20 μ l | vehicle or theophylline or appropriate concentration of test compound |
| 50 μ l | ³ H-CHA |

The tubes are incubated for 2 hours at 25°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml liquiscintillation cocktail, left to soak overnight and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 1 mM theophylline. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors (Bruns et al. 1986) precludes the simple calculation of K_i values by the Cheng-Prusoff equation.

MODIFICATIONS OF THE METHOD

Stiles et al. (1985) used ¹²⁵I-labeled N⁶-2-(4-aminophenyl)ethyladenosine as a selective ligand to probe the structure of A₁ receptors.

Lohse et al. (1987) described 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as a high affinity antagonist radioligand for A₁ adenosine receptors.

Klotz et al. (1989) described 2-chloro-N⁶-[³H]cyclopentyladenosine ([³H]CCPA) as a high affinity agonist radioligand for A₁ adenosine receptors.

Von Lubitz et al. (1995) studied the therapeutic implications of chronic NMDA receptor stimulation on adenosine A₁ receptors.

The partial agonism of theophylline-7-riboside on the adenosine A₁ receptor has been reported by Ijzerman et al. (1994).

Libert et al. (1992) reported the cloning and functional characterization of a human A₁ adenosine receptor.

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A.1.1.9

Adenosine A₂ Receptor Binding

PURPOSE AND RATIONALE

The A₂ receptor is a low-affinity binding site for adenosine (Daly et al. 1981). Activation of the A₂ receptor subtype by agonists mediates an increase in adenylate cyclase activity, while the A₁ receptor has the opposite effect. Although many of the physiological effects of adenosine seem to correlate with activity at the A₁ receptor, the effect on coronary blood flow correlates with activation of A₂ receptors (Hamilton et al. 1987).

This assay uses ³H-NECA (5'-N-ethylcarboxamido[8-³H]adenosine) to label A₂ receptors in rat striatum by the method described by Bruns et al. (1986). Comparison of data from this assay and the A₁ receptor assay provides a measure of selectivity for these two receptors.

PROCEDURE

Reagents

1. a) 0.5 M Tris buffer, pH 7.7
b) 0.05 M Tris buffer, pH 7.7
c) 0.05 M Tris buffer, pH 7.7, containing 12 mM CaCl₂ (final assay concentration: 10 mM)
2. Adenosine deaminase is obtained from Sigma Chemical Co.

Adenosine deaminase is added to 0.05 M Tris-HCl buffer, pH 7.7, containing 12 mM CaCl₂ for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.

3. 5'-N-Ethylcarboxamido[8-³H]adenosine (specific activity 23–40 mCi/mmol) is obtained from Amersham.

For IC₅₀ determinations: ³H-NECA is made up to a concentration of 80 nM and 50 μl is added to each tube. This yields a final concentration of 4 nM in the assay.

4. Cyclopentyladenosine (CPA) is obtained from Research Biochemicals Inc.

A 5 mM stock solution is made up in DMSO. 20 μl are added to each of 3 tubes for the determination of nonspecific binding, yielding a 100 μM final concentration in the assay.

Since [³H]NECA is not a specific ligand for A₂ receptors, CPA is added to all other tubes to mask the A₁ receptors at a final concentration of 50 nM.

5. Test compounds

For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2 × 10⁻⁵ to 2 × 10⁻⁸ M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 g for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellet is resuspended in 100 volumes of 0.05 M Tris buffer containing 10 mM CaCl₂ and 0.1 U/ml of adenosine deaminase.

Assay

| | |
|--------|--------------------------------------------------------------|
| 830 μl | tissue suspension |
| 100 μl | CPA |
| 20 μl | vehicle or CPA or appropriate concentration of test compound |
| 50 μl | ³ H-NECA |

The tubes are incubated at 25°C for 2 hours. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml Liquiscint scintillation cocktail, left to soak overnight and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 100 μM CPA. IC₅₀ values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors precludes the simple calculation of K_i values by the Cheng–Prusoff equation.

MODIFICATIONS OF THE METHOD

Jarvis et al. (1989) reported on [³H]CGS 21 680, a selective A₂ adenosine receptor agonist which directly labels A₂ receptors in rat brain. [³H]CGS 21 680 binding was greatest in striatal membranes with negligible specific binding obtained in rat cortical membranes.

Gurden et al. (1993) described the functional characterization of three adenosine receptor types.

Hutchinson et al. (1990) described 2-(arylalkylamino)adenosin-5'-uronamides as a new class of highly selective adenosine A₂ receptor ligands.

A_{2A} Adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells have been characterized with radioligand binding and by activation of adenylate cyclase (Hide et al. 1992).

Nonaka et al. (1994) reported on KF17837 ((E)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine), a potent and selective adenosine A₂ receptor antagonist.

The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist has been reported by Poucher et al. (1955).

Monopoli et al. (1994) described the pharmacology of the selective A_{2α} adenosine receptor agonist 2-hexynyl-5'-N-ethylcarboxamidoadenosine.

Jacobson et al. (1993) described structure-activity relationships of 8-styrylxanthines as A₂-selective adenosine antagonists.

Varani et al. (1996) reported pharmacological and biochemical characterization of purified A_{2a} adenosine receptors in human platelet membranes by [³H]-CGS 21680 binding.

Van der Ploeg et al. (1996) characterized adenosine A₂ receptors in human T-cell leukemia Jurkat cells and rat pheochromocytoma PC12 cells using adenosine receptor agonists.

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A.1.1.10

Adenosine A₃ Receptor Binding

PURPOSE AND RATIONALE

The A₃ adenosine receptor has been cloned and characterized by Zhou et al. (1992). A possible role in reproduction has been discussed. The role of central A₃ adenosine receptors may be the mediation of behavioral depressant effects (Jacobson et al. 1993). The

design of selective ligands of A₃ adenosine receptors and the therapeutic concepts including effects on locomotor activity, cardiovascular effects, effects in cerebral ischemia (von Lubitz et al. 1994), in cardiac preconditioning and as antagonists in inflammation and asthma has been discussed by Jacobson et al. (1995). Von Lubitz et al. (1995) noticed some anticonvulsive activity of the adenosine A₃ receptor selective agonist IB-MECA (N⁶-(3-iodobenzyl) adenosine-5'-N-methyl-carboxamide). Stimulation of the A₃ adenosine receptor facilitates release of allergic mediators in mast cells (Ramkumar et al. 1993) inducing hypotension in the rat (Hannon et al. 1995). A binding site model and structure-activity relationships for the rat A₃ adenosine receptor are described by van Galen et al. (1994).

PROCEDURE

Cell culture and membrane preparation

Chinese hamster ovary (CHO) cells stably expressing the rat A₃ adenosine receptor are grown in F-12 medium containing 10% fetal bovine serum and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively) at 37° in a 5% CO₂ atmosphere. When cells reach confluency, they are washed twice with 10 ml of ice-cold lysis buffer (10 mM EDTA, pH 7.4). After addition of 5 ml of lysis buffer, cells are mechanically scraped and homogenized in an ice-cold Dounce homogenizer. The suspension is centrifuged at 43,000 g for 10 min. The pellet is suspended in the minimum volume of ice-cold 50 mM Tris/10 mM MgCl₂/1 mM EDTA (pH 8.26 at 5°C) buffer required for the binding assay and homogenized in a Dounce homogenizer. Aminodeaminase (ADA, Boehringer Mannheim) is added to a final concentration of 3 units/ml and the suspension is incubated at 37°C for 15 min; the membrane suspension is subsequently kept on ice until use.

Radioligand binding assay

Binding of [¹²⁵I]APNEA (N⁶-2-(4-aminophenyl)-ethyladenosine) to CHO cells stably transfected with the rat A₃ adenosine receptor clone is performed according to Stiles et al. (1985). Assays are performed in 50/10/1 buffer in glass tubes and contain 100 µl of the membrane suspension, 50 µl of inhibitor. Incubations are carried out in duplicate for 1 h at 37°C and are terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester. Tubes are washed three times with 3 ml of buffer. Radioactivity is determined in a Beckman γ-counter. Non-specific binding is determined in the presence of 40 µM R-PIA = N⁶-[(R)-1-methyl-2-phenylethyl]adenosine.

EVALUATION

K_i values are calculated according to Cheng and Prusoff (1973), assuming a K_d for [125 I]APNEA of 17 nM.

MODIFICATIONS OF THE METHOD

125 I-4-aminobenzyl-5'-N-methylcarboxamidoadenosine has been recommended as a high affinity radioligand for the rat A_3 adenosine receptor (Olah et al. 1994).

Molecular cloning and functional expression of a sheep A_3 adenosine receptor has been reported by Linden et al. (1993).

G protein-dependent activation of phospholipase C by adenosine A_3 receptors in rat brain was reported by Abbracchio et al. (1995).

Molecular cloning and characterization of the human A_3 adenosine receptor was reported by Salvatore et al. (1993).

The differential interaction of the rat A_3 adenosine receptor with multiple G-proteins has been described by Palmer et al. (1995).

Baraldi and Borea (2000) described new potent and selective human adenosine A_3 receptor antagonists using radioligand binding studies to the human A_3 receptor.

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A.1.1.11**Inhibition of Adenosine Uptake in Human Erythrocytes****PURPOSE AND RATIONALE**

Adenosine regulates multiple physiological functions in animals and humans. It plays a potent neuromodulatory role mainly by inhibiting the presynaptic transmitter release, e. g. of glutamate and aspartate. It is released by synaptic stimulation and during hypoxia in the central and peripheral nervous system. Adenosine plays a neuroprotective role in hypoxia and ischemia since it reduces the excessive stimulation of the NMDA receptors. The use of adenosine uptake inhibitors has been proposed as a new therapeutic strategy for hypoxic/ischemic disease. Due to its vasodilatory action adenosine plays a key role in the regulation of coronary and cerebral blood flow. The rapid cellular uptake of adenosine by erythrocytes is a reason for the short duration of action of adenosine.

Human erythrocytes are used as a cellular model to detect adenosine uptake inhibitors. Erythrocytes are treated with test compound and thereafter incubated with 3 H-adenosine. The uptake of 3 H-adenosine is evaluated in relation to the untreated control group.

Dipyridamole is a potent inhibitor of adenosine uptake (IC_{50} of 3×10^{-7} M).

Standard compounds:

- theophylline
- dipyridamole (Persantin)
- propentofylline (HWA 285)

PROCEDURE

Materials and solutions

isotonic glycyl-glycine buffer, pH 7.4

| | |
|-------------------------------------------------------------------|----------|
| KCl | 5.0 mM |
| NaCl | 119.5 mM |
| MgCl ₂ | 2.0 mM |
| glycyl-glycine | 50 mM |
| Na ₂ HPO ₄ | 2.0 mM |
| 2-[³ H]-adenosine (specific activity 0.2 μCi/μmol) | 5 μM |

Buffer-washed fresh human erythrocytes are depleted of ATP by incubation in an isotonic glycyl-glycine buffer at 37°C. Aliquots of the erythrocyte suspensions are incubated for 2 min in fresh glycyl-glycine buffer solution containing additional 10 mM glucose and test- or standard compound. In screening assays, test compounds are added at a concentration of 5×10^{-4} M. Drugs showing an effect in this assays, are further tested at a concentration range of 10^{-5} – 5×10^{-4} M to determine IC_{50} values (triplicate samples for each concentration).

The suspension is then incubated with 5 μM radioactively labelled 2-[³H]-adenosine for 30 s. The adenosine uptake is stopped by adding cold buffer (4°C) containing 5 μM adenosine, 10 μM glucose and 7.4 μM dipyridamole. After centrifugation, the tritium radioactivity is determined in the supernatant.

EVALUATION

The percent change of ³H-adenosine uptake relative to the vehicle control group is determined. The ³H-adenosine uptake of the control group is taken as 100%; subsequent results are expressed as percentages of this.

IC_{50} values are determined by plotting the percent inhibition against test compound concentration; IC_{50} is defined as the dose of drug leading to a 50% inhibition of adenosine uptake.

Statistical evaluation is performed by means of the Student's *t*-test.

Standard data:

- IC_{50} of dipyridamole 3×10^{-7} M

MODIFICATIONS OF THE METHOD

Marangos et al. (1982), Verma and Marangos (1985) recommended [³H]nitrobenzylthioinosine binding as

a probe for the study of adenosine uptake sites in brain of various species. The highest density of binding sites were found in the caudate and hypothalamus of human and rat brain.

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A.1.1.12

Inhibition of Vasopeptidases

GENERAL CONSIDERATIONS

Vasopeptidase inhibitors (VPIs) inhibit both angiotensin converting enzyme (ACE) and neprilysin (NEP) and can thus reduce the activity of the renin-angiotensin system and potentiate the vasodilatory, natriuretic and antiproliferative effects of bradykinin and natriuretic peptides (Burnett 1999; Bralet and Schwartz 2001). Combined inhibition of neutral endopeptidase 24.11 (NEP) and ACE is a candidate therapy for hypertension and cardiac failure (Duncan et al. 1999). Heath et al. (1995) described the quantification of a dual ACE-I-converting enzyme-neutral endopeptidase inhibitor and the active thiol metabolite in dog plasma by high-performance liquid chromatography with ultraviolet absorption detection. Dumoulin et al. (1995) studied the metabolism of bradykinin by the rat coronary vascular bed and found that combined treatment with the ACE inhibitor enalaprilate and the NEP inhibitor retrothiorphan reduced bradykinin degradation to lower values than enalaprilate alone.

Hubner et al. (2001) reported *in-vitro* and *in-vivo* inhibition of rat neutral endopeptidase and ACE with the vasopeptidase inhibitor gemopatriat. Dumoulin et

al. (2001) compared the effects of a vasopeptidase inhibitor with those of neutral endopeptidase and ACE inhibitors on bradykinin metabolism in the rat coronary bed.

Crackower et al. (2002) described type-2 ACE (ACE2) as an essential regulator of heart function. In three different rat models of hypertension, ACE2 messenger RNA and protein expression were markedly reduced. Targeted disruption of ACE2 in mice resulted in a severe cardiac contractility defect.

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A.1.1.12.1

Inhibition of the Angiotensin-Converting Enzyme in Vitro

PURPOSE AND RATIONALE

An *in vitro* system can be used to screen potential angiotensin-converting enzyme inhibitors. Fluorescence generated by an artificial substrate in presence or absence of the inhibitor is measured to detect inhibitory activity.

PROCEDURE

Reagents

- 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl
- 10 mM potassium phosphate buffer, pH 8.3
- Substrate: O-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (molecular weight 482) (Bachem Gentec. Inc., Torrance, California, USA)
 - stock solution: 10 mg substrate in 10 ml 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl
 - working solution: 2 ml stock solution is added to 18 ml 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl; final concentration in the assay is 170.2 μ M.
- Test compounds

Compounds are made up to a concentration of 1 mM in 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl or 10% methanol in Tris/NaCl if insoluble in aqueous buffer alone. This will give a final concentration in the assay of 0.1 mM. If inhibition is seen, further dilution in Tris/NaCl should be made.

Enzyme preparation

Lung tissue from 10 rats is diced and homogenized in a blender with 3 pulses of 15 s each. The homogenate is centrifuged at 5000 g for 10 min. The pellet is discarded, the supernatant is dialyzed against three 1 liter changes of 10 mM potassium phosphate buffer, pH 8.3 overnight in the cold and then centrifuged at 40,000 g for 20 min. The pellet is discarded, 390 mg $(\text{NH}_4)_2\text{SO}_4$ is added for each ml of supernatant. This will give 60% saturation. The solution is stirred on ice for 15 min. The pellet formed is dissolved in 15 ml potassium phosphate buffer, pH 8.3 and dialyzed against the same buffer overnight in the cold with three 1 liter changes. Some protein will precipitate during dialysis. The suspension is centrifuged at 40,000 g for 20 min and the supernatant is discarded. The final solubilized enzyme preparation can be aliquoted and stored at -20°C at least 6 months.

Enzyme inhibition studies

- Enzyme activity is measured with a Perkin Elmer LS-5 Fluorescence Spectrophotometer or equivalent at an excitation wavelength of 357 nm and an emission wavelength of 424 nm.
- Enzyme assay
 - 50 μ l vehicle or inhibitor solution and 40 μ l enzyme are preincubated for 5 min, then 410 μ l substrate working solution is added.

Samples are mixed by drawing fluid back up into the pipette and by pipetting into the cuvette. For the initial

control run of the day, the auto zero is pushed immediately after placing the sample in the cuvette.

EVALUATION

The individual fluorescence slope is measured and % inhibition is calculated as follows:

$$\begin{aligned} & \% \text{ inhibition} \\ & = \% \text{ inhibition} \\ & = \left(100 - \frac{\text{slope in presence of inhibitor}}{\text{control slope}} \right) \times 100 \end{aligned}$$

Inhibitor concentrations on either side of the IC_{50} should be tested to generate a dose-response curve. The IC_{50} is calculated using Litchfield-Wilcoxon log-probit analysis.

Standard data:

- IC_{50} values for inhibition of angiotensin I-converting enzyme
- Compound IC_{50} [M]
- Captopril 6.9×10^{-9}

MODIFICATIONS OF THE METHOD

Other assays use the cleavage of hippuric acid from tripeptides (Hip-Gly-Gly or Hip-His-Leu) whereby hippuric acid is either tritium labelled or determined spectrophotometrically (Cushman and Cheung 1969, 1971; Friedland and Silverstein 1976; Santos et al. 1985; Hecker et al. 1994).

Bünning (1984) studied the binding and inhibition kinetics of ramipril and ramiprilate (Hoe 498 diacid) with highly purified angiotensin converting enzyme using furanacryloyl-Phe-Gly-Gly as substrate.

The importance of tissue converting enzyme inhibition in addition to inhibition in plasma has been verified in several studies (Unger et al. 1984, 1985; Linz and Schölkens 1987).

Eriksson et al. (2002), Oudit et al. (2003), and Danilczyk et al. (2003, 2004) discussed the role of the homolog of angiotensin-converting enzyme ACE2 in cardiovascular physiology. ACE2 appears to negatively regulate the renin-angiotensin system and cleaves Ang I and Ang II into the inactive Ang 1-9 and Ang 1-7. ACE2 differs in its specificity and physiological role from ACE.

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A.1.1.12.2

Inhibition of Neutral Endopeptidase (Neprilysin)

PURPOSE AND RATIONALE

Neutral endopeptidase cleaves various peptides, such as enkephalins, kinins, chemotactic peptide, atrial natriuretic factor, and substance P. Reviews on neutral endopeptidase 24.11 (enkephalinase) were given by Erdös and Skidgel (1989) and by Roques et al. (1993). Structural requirements were investigated by Santos et al. (2002).

Several enzymatic assays have been developed for measuring neutral endopeptidase (NEP) activity, such as radiolabeled methods (Vogel and Altstein 1977; Llorens et al. 1982) and colorimetric assays (Almenoff et al. 1981; Almenoff and Orłowsky 1984); fluorometric assay (Florentin et al. 1984; Goudreau et al. 1994). Burell et al. (1997) and Hubner et al. (2001) used the selective NEP inhibitor radioligand ^{125}I -labelled RB104.

Cavalho et al. (1995, 1996) described a highly selective assay for neutral endopeptidase based on the cleavage of a fluorogenic substrate related to Leu-enkephalin.

PROCEDURE

A recombinant soluble form of NEP (rNEP) was expressed using a baculovirus/insect-cell system and purified by immunoaffinity.

The substrate (10 nmol) was incubated with rNEP (100 ng) in a final volume of 100 μl of 50 mM Tris-HCl buffer, pH 7.4, at 37°C for 30 min. For the inhibition assays, the enzyme was preincubated with 1 μM thiorphan or 1 μM captopril for 20 min before its incubation with the substrate. The reaction was stopped by heating for 5 min at 100°C. After centrifugation at 10,000 g for 10 min, the supernatant fraction was injected into an HPLC column and eluted with a 20%–40% gradient of acetonitrile containing 0.05% trifluoroacetic acid over a period of 30 min, at a flow rate of 1 ml/min. The substrate and products, detected by both UV absorbance (220 nm) and fluorescence ($\lambda_{\text{em}} = 420 \text{ nm}$, $\lambda_{\text{ex}} = 320 \text{ nm}$) with the detectors arranged in series, were collected to identify the cleavage site by amino acid analysis.

EVALUATION

Kinetic parameters for the NEP-catalyzed hydrolysis were determined from the double-reciprocal Lineweaver-Burk plots.

MODIFICATIONS OF THE METHOD

Sulpizio et al. (2004) described the determination of **NEP activity in tissues** after *in vivo* treatment of rats with ACE inhibitors. After sacrifice of the animals, approximately 250 mg of kidney tissue was homogenized in 6 volumes of 0.1 M KH_2PO_4 , pH 8.3, 0.3 M NaCl, and 1 μM ZnSO_4 , using a Teflon-glass motor-driven pestle. NEP activity was measured by adding 35 μl of homogenate to wells containing 5 μl buffer or 10 phosphoramidon. Next, 10 μl of 2.5 mM *N*-dansyl-D-alanyl-p-nitro-phe-gly substrate (Florentin et al. 1984) was added to each sample to yield a 0.5 mM final

concentration and incubated for 4 min at 37°C. Subsequently, 100 μl of 10% TCA was added and plates were centrifuged to pellet precipitated proteins. Then 50 μl of supernatant was added to 100 μl of 100% ethanol and 50 μl of 1 N NaOH in a black fluorometric plate. After 10 min, plates were read at 590 nm emission, 320 nm excitation in a fluorometer.

Zhang et al. (1994) described an ELISA for the neuropeptide endopeptidase 3.4.24.11 in human serum and leukocytes.

Gros et al. (1989) studied the protection of atrial natriuretic factor against degradation and the diuretic and natriuretic responses after *in vivo* inhibition of enkephalinase (EC 3.4.24.11) by acetorphan. Increased tissue neutral endopeptidase 24.11 activity in spontaneously hypertensive hamsters was reported by Vishwanata et al. (1998). Graf et al. (1998) studied regulation of neutral endopeptidase 24.11 in human vascular smooth muscle cells by glucocorticoids and protein kinase C.

Pham et al. (1992) described the effects of a selective endopeptidase inhibitor on renal function and blood pressure in conscious normotensive Wistar and hypertensive DOCA-salt rats.

NEP is involved in organ systems other than the cardiovascular system, for example the brain and lung.

Ratti et al. (2001) studied the correlation between neutral endopeptidase (NEP) in serum and the degree of bronchial hyperreactivity.

Shirotani et al. (2001) found that neprilysin degrades both amyloid β peptides 1–40 and 1–42 very rapidly and efficiently. Newell et al. (2003) found that thiorphan-induced neprilysin inhibition raises amyloid β levels in rabbit cortex and cerebrospinal fluid.

Facchinetti et al. (2003) described the ontogeny, regional and cellular distribution of metalloprotease neprilysin 2 (**NEP2**) in the rat in comparison with neprilysin and endothelin-converting enzyme-1.

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A.1.1.13

Quantitative Autoradiographic Localization of Angiotensin-Converting Enzyme

PURPOSE AND RATIONALE

Cardiac angiotensin converting enzyme can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized *in vitro* autoradiography (Kohzuki et al. 1996)

PROCEDURE

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see A.3.2.2). After various time intervals (1–8 months) the animals are decapitated, the hearts rapidly removed, and snap-frozen in isopentane at –40°C. Frozen section (20 μm) are cut in a cryostat at –20°C. The sections are thaw-mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4°C and then stored at –80°C.

Quantitative autoradiography

Radioligand: MK351A is a tyrosyl derivative of lisinopril, a potent competitive inhibitor of ACE. MK351A is iodinated by the chloramine T method and separated free from ¹²⁵I by SP Sephadex C25 column chromatography.

¹²⁵I-MK351A binding: The sections are preincubated in 10 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl and 2% bovine serum albumin for 15 min at 20°C. The sections are then incubated with 11.1 KBq/ml ¹²⁵I-MK351A in the same buffer for 60 min at 20°C. Nonspecific binding is determined in the presence of 10^{–6} mol/L MK351A or lisinopril. Binding isotherms are determined using a set of serial sections incubated with 10^{–12}–10^{–6} mol/L lisinopril for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h

at room temperature. After exposure, the sections are fixed in formaldehyde and stained with haematoxylin and eosin. The optical density of the X-ray films is quantified using an imaging device controlled by a personal computer.

EVALUATION

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm² with reference standards maintained through the procedure. The apparent binding site concentration (B_{\max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle and the non-infarcted area in the left ventricle are estimated by an iterative non-linear model-fitting computer program LIGAND (Munson and Rodbard 1980).

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A.1.1.14

Angiotensin Antagonism

The renin-angiotensin-aldosterone hormonal axis is the major long-term control for regulation of both arterial blood pressure and sodium balance. It supports normotension or hypertension via angiotensin vasoconstriction and angiotensin plus aldosterone-induced renal sodium retention (Laragh 1993; Unger and Schölkens 2004).

Volpe et al. (1995) Wagner et al. (1996) showed that regulation of aldosterone biosynthesis by adrenal renin is mediated through AT₁ receptors in renin transgenic rats.

Easthope and Jarvis (2002) reviewed pharmacological, pharmacokinetic and clinical data on the angiotensin II antagonist candesartan cilexetil.

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A.1.1.14.1

Angiotensin II Receptor Binding

PURPOSE AND RATIONALE

Angiotensin II receptor subtypes, AT₁ and AT₂, have been identified by structurally dissimilar antagonists, by different distribution in organs of various species and with specific radioligands (Chiu et al. 1989, 1990, 1992, 1993; Chang and Lotti 1991; Gibson et al. 1991; Chansel et al. 1992; Steckelings et al. 1992; Aiyar et al. 1993; Barnes et al. 1993; Bossé et al. 1993; Bottari et al. 1993; Dzau et al. 1993; Feuillan et al. 1993; van Meel et al. 1993; Alexander et al. 2001). These two types of receptors have been cloned (Sasaki et al. 1991; Murphy et al. 1991; Mukoyama et al. 1993; Kambayashi et al. 1993). Two other mammalian receptors named AT₃ and AT₄ have been described (de Gasparo et al. 1998).

The functional correlates of angiotensin II receptors have been discussed by Timmermans et al. (1992, 1993; Bernstein and Berk 1993). Most effects of angiotensin are mediated via the AT₁ receptors, but a possible role of angiotensin II subtype AT₂ receptors in endothelial cells and isolated ischemic rat hearts has been suggested (Wiemer et al. 1993a, b). Clearance studies in dogs indicated that the angiotensin type 2 receptor may be related to water handling in the kidney (Keiser et al. 1992).

Evidence for AT₁ receptor subtypes (AT_{1A} and AT_{1B}) has been reported (Iwai and Inagami 1992; Kakar et al. 1992; Balmforth et al. 1994; Matsubara et al. 1994; Bauer and Reams 1995; de Gasparo et al. 1998).

Chai et al. (2004) described the properties of the angiotensin IV/AT₄ receptor.

The assay described below is used to determine the affinity of test compounds to the angiotensin II receptor by measuring their inhibitory activity on the bind-

ing of ^3H -angiotensin II to a plasma membrane preparation from rat or bovine adrenal cortex.

PROCEDURE

Fresh bovine adrenal glands are obtained from the local slaughter house. For rat adrenal glands, male Sprague-Dawley rats weighing 250–300 g are sacrificed. The adrenals are separated from fat tissue and the medullae removed. The cortices are minced and homogenized in 5 mM Tris buffer containing 1 mM MgCl_2 and 250 mM sucrose, pH 7.4, using a chilled Potter homogenizer. The homogenate is centrifuged at 3000 g and 4°C for 10 min. The supernatant is recentrifuged at 39,000 g and 4°C for 10 min. The pellets are resuspended in 75 mM Tris buffer containing 25 mM MgCl_2 , pH 7.4, and recentrifuged twice at 39,000 g and 4°C for 10 min. After the last centrifugation, the pellets are suspended in 75 mM Tris buffer containing 25 mM MgCl_2 and 250 mM sucrose, pH 7.4. Samples of 0.5 ml are frozen in liquid nitrogen and stored at -70°C .

In the competition experiment, 50 μl ^3H -angiotensin II (one constant concentration of $0.5\text{--}1 \times 10^{-9}$ M), and 50 μl test compound (6 concentrations, $10^{-5}\text{--}10^{-10}$ M) and 100 μl membrane suspension from rat or bovine adrenal cortex (approx. 250 mg wet weight/ml) per sample are incubated in a bath shaker at 25°C for 60 min. The incubation buffer contains 50 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, 5 mM MgCl_2 and 0.2% bovine serum albumin, pH 7.4.

Saturation experiments are performed with 12 concentrations of ^3H -angiotensin II ($15\text{--}0.007 \times 10^{-9}$ M). Total binding is determined in the presence of incubation buffer, non-specific binding is determined in the presence of non-labeled angiotensin II (10^{-6} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION OF RESULTS

The following parameters are calculated:

- total binding of ^3H -angiotensin II
- non-specific binding: binding of ^3H -angiotensin II in the presence of mepyramine or doxepine
- specific binding = total binding – non-specific binding
- % inhibition of ^3H -angiotensin II binding: $100 - \frac{\text{specific binding}}{\text{percentage of control value}}$

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^3H -angiotensin II versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

MODIFICATIONS OF THE METHOD

Olins et al. (1993) performed competition studies in rat uterine smooth muscle membranes and rat adrenal cortex membranes using [^{125}I] labeled angiotensin II.

Membranes from cultured rat aortic smooth muscle cells and from human myometrium were used for binding studies with [^{125}I] labeled angiotensin II by Criscone et al. (1993).

Wiener et al. (1993) used membrane preparations from rat lung and adrenal medulla for binding studies with [^{125}I] labeled angiotensin II.

Bradbury et al. (1993) used a guinea pig adrenal membrane preparation to study nonpeptide angiotensin II receptor antagonists.

Cazaubon et al. (1993) prepared purified plasma membranes from rat livers for [^{125}I] AII binding assays.

Noda et al. (1993) described the inhibition of rabbit aorta angiotensin II (AII) receptor by a non-peptide AII antagonist.

Kushida et al. (1995) tested AT II receptor binding in particulate fractions of rat mesenteric artery and rat adrenal cortex and medulla with ^{125}I -AT II.

Chang et al. (1995) used rabbit aorta, rat adrenal and human AT₁ receptors in CHO cells and AT₂ receptors from rat adrenal and brain to characterize a non-peptide angiotensin antagonist.

Aiyar et al. (1995) tested inhibition of [^{125}I] angiotensin II or [^{125}I] angiotensin II (Sar¹,Ile⁸) binding in various membrane and cell preparations, such as rat mesenteric artery, rat adrenal cortex, rat aortic smooth muscle cell, human liver, recombinant human AT₁ receptor, bovine cerebellum, and bovine ovary.

Caussade et al. (1995) tested [^{125}I]Sar¹,Ile⁸-angiotensin II binding to rat adrenal membranes and rat aortic smooth muscle cells.

Using [^{125}I]Sar¹,Ile⁸-angiotensin II as radioligand, de Gasparo and Whitebread (1995) compared the affinity constants of valsartan and losartan in liver and adrenal of rat and marmoset, human adrenal and in rat aortic smooth muscle cells.

Webb et al. (1993) transfected the vascular angiotensin II receptor cDNA (AT_{1A}) into Chinese hamster ovary cells to generate the stable cell line CHO-AT_{1A} and recommended these cells as a useful model

to study AT_{1A} receptor domains, which are critical to signaling pathways.

Kiyama et al. (1995) used COS cells transfected with a cDNA encoding a human AT₁ angiotensin II receptor to evaluate nonpeptide angiotensin II receptor antagonists.

Mizuno et al. (1995) used bovine adrenal cortical membranes, Nozawa et al. (1997) membrane fractions from rat aorta, bovine cerebellum and human myocardium and [¹²⁵I]angiotensin II as radioligand.

Renzetti et al. (1995a, b) used membranes from rat adrenal cortex and bovine cerebellum for binding assays with [³H]angiotensin II as radioligand.

Inter-species differences in angiotensin AT₁ receptors were investigated by Kawano et al. (1998).

The angiotensin II receptor subtype having a high affinity for losartan has been designated angiotensin AT₁ receptor and the receptor having a high affinity for PD123177 (1-(3-methyl-4-aminophenyl) methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imidazo[3,5-c]pyridine-6-carboxylic acid) as angiotensin AT₂ receptor (Bumpus et al. 1991; Nozawa et al. 1994; Chang et al. 1995).

In order to determine affinity for the angiotensin AT₁ subtype in a radioligand binding assay with [¹²⁵I]-sarcosine¹, isoleucine⁸ angiotensin II, Chang and Lotti (1991), Chang et al. (1995), Wong et al. (1995) incubated membranes of tissues with both AT₁ and AT₂ receptors in the presence of 1 μM PD121981 (which occupied all the AT₂ binding sites) and for the angiotensin AT₂ subtype in the presence of 1 μM losartan (which occupied all the AT₁ binding sites).

Hilditch et al. (1995) used membranes from rat livers and [³H]-AT II for the determination of binding affinity at AT₁ receptors, or membranes from bovine cerebellum and [¹²⁵I]-Tyr⁴-AT II for AT₂ receptors.

Lu et al. (1995) studied the influence of freezing on the binding of [¹²⁵I]-sarcosine¹, isoleucine⁸ angiotensin II to angiotensin II receptor subtypes in the rat. The results suggested that studies of AII receptor subtypes that involve freezing of the tissue underestimate the density and affinity of the AT₁ receptor subtype.

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- addition of 1 μM losartan. Binding of [¹²⁵I]Ang II to membranes was conducted in a final volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA, homogenate corresponding to 10 mg of the original tissue weight, [¹²⁵I]Ang II (80,000–85,000 cpm, 0.03 nM), and variable concentration of test substance. Samples were incubated at 25°C for 1.5 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets, which had been pre-soaked overnight with 0.3% polyethylamine, using a Brandel cell harvester. The filters were washed with 3 × 3 ml of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a γ-counter. All determinations were performed in triplicate.

EVALUATION

The characteristics of the Ang-II-binding AT₂ receptor were determined by using six different concentrations (0.03–5 nmol/l) of the labeled [¹²⁵I]-Ang II. Non-specific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the non-specific binding from the total bound [¹²⁵I]-Ang II. IC₅₀ was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK).

MODIFICATIONS OF THE METHOD

Whitebread et al. (1991) described the radioligand CGP 42112A as a high-affinity and highly selective ligand for the characterization of angiotensin AT₂ receptors. Heemskerk and Saavedra (1995) performed quantitative autoradiography of Ang II AT₂ receptors with [¹²⁵I]CGP 42112.

Heerding et al. (1997) performed mutational analysis of the Ang II type 2 receptor in order to study the contribution of conserved extracellular amino acids.

Hoe et al. (2003) reported the molecular cloning, characterization, and distribution of the gerbil Ang II AT₂ receptor.

Utsunomiya et al. (2005) described Ang II AT₂ receptor localization in cardiovascular tissues by its antibody developed in AT₂ gene-deleted mice.

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A.1.1.14.2

AT₂ Receptor Binding

PURPOSE AND RATIONALE

In addition to the AT₁ receptor, the AT₂ receptor has attracted increasing interest (Gallinat et al. 2000; Nouet and Nahmias 2000). Steckelings et al. (2005) reviewed the knowledge of AT₂ receptor distribution, signaling and function with an emphasis on growth/anti-growth, differentiation, and the regeneration of neuronal tissue.

Wan et al. (2004a, 2004b) described a porcine myometrial membrane AT₂ receptor assay.

PROCEDURE

Myometrial membranes are prepared from porcine uteri. Nielsen et al. (1997) found that in myometrium from non-pregnant sows, the Ang II receptors were almost exclusively AT₂ receptors. A presumable interference by binding to AT₁ receptors was blocked by

- bution of conserved extracellular amino acids. *Regul Pept* 72:97–103
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A.1.1.14.3

Angiotensin II Induced Contraction in Isolated Rabbit Aorta

PURPOSE AND RATIONALE

The isolated rabbit aorta has been used to evaluate angiotensin II agonists (Liu 1993) and angiotensin II antagonists (Chang et al. 1992, 1994; Noda et al. 1993; Aiyar et al. 1995; Cirillo et al. 1995; Kushida et al. 1995; Mochizuki et al. 1995; Renzetti et al. 1995; Wong et al. 1995; Hong et al. 1998; Kawano et al. 1998).

PROCEDURE

New Zealand White male rabbits weighing 2–3 kg are sacrificed and exsanguinated. The thoracic aorta is removed and cleaned from adherent fat and connective tissue. The vascular endothelium is removed by gently rubbing the intimal surface of the vessel. Spiral aortic strips (2–3 mm wide and 30 mm long) are prepared and mounted in 5 ml organ baths containing Krebs-Henseleit solution (120 mM NaCl, 4.7 mM KCl, 4.7 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM

CaCl₂, 25 mM NaHCO₃, glucose 10 mM, pH 7.4). The organ baths are kept at 37°C and gassed continuously with 95% O₂/5% CO₂. Strips are attached to isometric transducers connected to a polygraph and a resting tension of 1 g is applied to each strip. Changes in contraction are analyzed with a digital computer. Aortic strips are allowed to equilibrate for 1 h and washed every 15 min. Two consecutive contractile-response curves to cumulative addition of AII (0.1–300 mM) are constructed. After each curve the strips are washed 4 times and allowed to relax to the baseline tension. Afterward, each strip is incubated for 30 min with the vehicle or with a single concentration of the antagonist (1–10–100–1000 mM) before a third concentration-response curve to angiotensin II is obtained.

EVALUATION

The result of each concentration is expressed as a percentage of maximum response to AII. The pA₂ and pD' values are calculated (van Rossum 1963).

MODIFICATIONS OF THE METHOD

Isolated guinea pig aortas were used by Mizuno et al. (1995).

Cirillo et al. (1995) evaluated the antagonism against AII-induced vasoconstriction in rat isolated perfused kidney.

Chang et al. (1992, 1994) determined AII-induced aldosterone release in rat adrenal cells and AII-induced [³H]inositol phosphate accumulation in cultured rat aorta smooth muscle cells.

Shibouta et al. (1993) described the pharmacological profile of a highly potent and long-acting Ang II receptor antagonist and its prodrug.

Ojima et al. (1997) studied the mechanisms of the insurmountable antagonism of candesartan, an angiotensin AT₁ receptor antagonist, on Ang-II-induced rabbit aortic contraction in contraction and binding studies.

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A.1.1.14.4

Angiotensin II Antagonism *in Vivo*

PURPOSE AND RATIONALE

The effect of ATII antagonists on blood pressure has been measured in anesthetized (Olins et al. 1993; Beauchamp et al. 1995; Kawano et al. 1998), in pithed (Cazes et al. 1995; Christophe et al. 1995; Cirillo et al. 1995; Deprez et al. 1995; Häuser et al. 1998) and in conscious (Junggren et al. 1996; Nozawa et al. 1997; Shibasaki et al. 1997; Hashimoto et al. 1998) normotensive and hypertensive rats.

PROCEDURE

Male Sprague-Dawley rats are anesthetized with 100 mg/kg i.p. Inactin and placed on servo-controlled heating pads to maintain body temperature between 37°C and 38°C. PE50 catheters are implanted in the femoral artery and vein to measure arterial blood pressure and administer compounds, respectively. A catheter is placed in the trachea to ensure airway patency. Arterial pressure is measured continuously by connecting the arterial catheter to transducer coupled to a Gould pressure transducer. The output is recorded on a polygraph. Mean arterial pressure is derived electronically. After a 30–45 min stabilization period, autonomic transmission is blocked by treatment with mecamylamine (3 mg/kg i.v.) and atropine (0.4 mg/kg i.v.). After arterial pressure has stabilized, angiotensin is infused i.v. in isotonic saline with a syringe pump. When the pressure response to angiotensin has stabilized, angiotensin II antagonists are given in increasing doses. The doses are given intravenously in a cumulative fashion, i. e., the next highest dose is given at the time of maximum response to the prior dose.

EVALUATION

Data are presented as percent inhibition of the angiotensin pressor response to each dose of the antagonists and plotted against the log of the cumulative doses of antagonist. Linear regression is used to calculate the dose at which the response to angiotensin is inhibited 50% (*ID*₅₀) for each rat. Means ±SEM are calculated.

MODIFICATIONS OF THE METHOD

Olins et al. (1993), Cirillo et al. (1995) determined also the antihypertensive effects in conscious spontaneously hypertensive rats and in conscious sodium-deficient dogs.

Stasch et al. (1997) studied the long-term blockade of the angiotensin II receptor in renin transgenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats.

Nishioka et al. (1998), Richter et al. (1998) used the (mRen-2)27 transgenic (Tg⁺) rat, a hypertensive model dependent on increased expression of the renin angiotensin system, to explore the role of angiotensin AT₂ receptors in the control of cardiovascular and renal excretory function.

Simoes e Silva et al. (1998) evaluated the effects of chronic administration of an angiotensin antagonist on diuresis and natriuresis in normotensive and spontaneously hypertensive rats.

Kai et al. (1998) examined the effects of an angiotensin II type I antagonist on cardiac hypertrophy and nephropathy using Tsukuba hypertensive mice (THM) carrying both human renin and angiotensinogen genes.

Kivlighn et al. (1995a, b), Gabel et al. (1995), studied angiotensin II antagonists in conscious rats, dogs, rhesus monkeys and chimpanzees.

Keiser et al. (1995) studied arterial blood pressure in conscious renal hypertensive rats, conscious sodium-depleted dogs, conscious sodium-depleted monkeys and conscious renal hypertensive monkeys.

Kim et al. (1997) examined the effects of an angiotensin AT₁ receptor antagonist on volume overload-induced cardiac gene expression in rats. Cardiac volume overload was prepared by abdominal aortocaval shunt. Cardiac tissue mRNA was measured by Northern blot analysis with specific probes.

Yamamoto et al. (1997), Ogilvie et al. (1998), studied angiotensin II receptor antagonists in acute heart failure induced by coronary artery ligation in anesthetized dogs and in chronic heart failure induced by left ventricular rapid-pacing in conscious dogs.

Massart et al. (1998) evaluated the cumulative hypotensive effects of angiotensin II- and endothelin-1-receptor antagonists in a model renovascular hypertension in dogs.

Hayashi et al. (1997) examined the hemodynamic effects of an angiotensin II type I receptor antagonist in rats with myocardial infarction induced by coronary ligation.

Kivlighn et al. (1995c) studied the effects of a non-peptide that mimics the biological actions of angiotensin II in anesthetized rats.

Huckle et al. (1996) evaluated angiotensin II receptor antagonists for their ability to inhibit vascular intimal thickening in a porcine coronary artery model of vascular injury.

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A.1.1.15

Renin-Inhibitory Activity Using Human Kidney Renin and a Synthetic Substrate

PURPOSE AND RATIONALE

In contrast to other enzymes, renin shows a rather high species specificity. To be relevant for humans human renin has to be used. One of the reasons that human renin is specific for human angiotensinogen lies in the sequence of human angiotensinogen itself. Inhibition of renin is measured by angiotensinogen formed in the presence of angiotensinase inhibitors. The following procedure is used to determine the effect of potential renin inhibitors on purified human kidney renin without interference from plasma proteins or lipids.

PROCEDURE

The synthetic substrate represents the first fourteen amino acids of the N-terminus of human angiotensinogen: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn. The assay mixture is composed of phosphate buffer (pH 7.5), bovine serum albumin, 3 mM EDTA, 0.01 mM phenylmethylsulfonyl fluoride (PMSF), 0.002% Genapol PF 10, test compound (dissolved in DMSO), substrate (3 μM) and purified human kidney renin (Calbiochem GmbH, Frankfurt/M., Germany; cat. no. 553861). The mixture is incubated for two hours at 37°C. Then the reaction is stopped by transfer of 450 μl into preheated (95°C) Eppendorf tubes. The amount of angiotensin I liberated is measured by RIA (Renin MAIA kit, Sero Diagnostika GmbH, Freiburg, Germany).

Human angiotensinogen (0.2 μM) may be used as a substrate instead of the tetradecapeptide. The pH value of the incubation mixture may be lowered to 6.0 by using a maleic acid buffer; this results in higher renin activity. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) may be substituted for phosphate in the pH 7.5 buffer.

EVALUATION

Renin activity, i.e. angiotensin I production (ng/ml × 2 h), is corrected for an angiotensin I-like immunoreactivity which can be measured in the assay samples even in the absence of added renin. IC₅₀ values are determined from a plot of renin activity (as per cent of control) vs. molar concentration of the test compound.

MODIFICATIONS OF THE METHOD

Wang et al. (1993) described a continuous fluorescence assay of renin activity employing a new fluorogenic peptide substrate.

Inhibition of plasma renin activity in blood samples from various species can be determined in order to evaluate the species specificity of a renin inhibitor (Linz et al. 1994)

Blood samples are obtained from dogs, sheep and rhesus monkeys by venipuncture. Wistar rats and guinea pigs are anesthetized with Nembutal (60 mg/kg intraperitoneally) and the blood is collected by puncture of the abdominal aorta. Human blood is collected from volunteers (Donafix blood collecting set, Braun Melsungen AG, Melsungen, FR Germany) in cooled bottles. All blood samples are anticoagulated with Na-EDTA (final concentration 10–15 mM). The renin is dissolved in DMSO as 10^{-2} M stock solution and diluted before each experiment in DMSO. The endogenous formation of ANG I in plasma during incubation at 37°C is determined as the measure of renin activity. Generation and quantitation of ANG I are performed using a commercial radioimmunoassay kit (Renin-MAIA, Serono Diagnostika GmbH, Freiburg, FR Germany). Plasma samples are thawed on ice and centrifuged after addition of 100 µl PMSF solution (kit) per 10 ml. The assay mixture contains 450 µl plasma plus 1% (v/v) PMSF solution, 45 µl buffer (phosphate buffer, pH = 7.4, + 10^{-5} M ramiprilate) and 5 µl renin inhibitor solution (diluted in DMSO as required) or pure DMSO for controls. The assay is incubated for an appropriate time (2–3 hours) at 37°C. ANG I is measured in 100 µl samples (triplicate determinations). Basal ANG I immunoreactivity of the plasma is determined from an unincubated control assay (0°C). This pre-incubation value is subtracted from all measurements. The renin activity in the presence of the renin inhibitor is calculated as percent activity in relation to control samples containing only DMSO. The IC_{50} value is determined from a semilogarithmic plot of percent renin activity versus concentration of the renin inhibitor.

Wood et al. (1990) determined the activity of a synthetic renin inhibitor against rat, mouse, dog, guinea pig, rabbit, cat, marmoset and human renin using plasma pools from these species. Plasma from each species was collected using EDTA as an anticoagulant. Samples of plasma were incubated at 37°C in the presence or absence of varying concentrations of test compound. The ANG I formed was measured by radioimmunoassay.

Shibasaki et al. (1991) used squirrel monkeys to study the *in vivo* activity of a specific renin inhibitor after intravenous and oral application.

Bohlender et al. (1996) reconstructed the human renin-angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using Alzet pumps.

Salimbeni et al. (1996) tested the *in vitro* inhibition of human plasma renin activity by two synthetic angiotensinogen transition state analogues.

Wood et al. (2003) determined the inhibitory potency of an orally effective renin inhibitor *in vitro* against human renin. Human recombinant renin (0.33 ng/ml) was incubated with a synthetic tetradecapeptide substrate (TDP, 13.33 µM) corresponding to the 14 terminal amino acids of human angiotensinogen, in 0.33 M Tes buffer, pH 7.2, containing 1% human serum albumin and 0.1% neomycin sulphate for 1 h at 37°C. The enzymatic reaction was stopped by adding 1 ml ice-cold 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1% HSA. The angiotensin generated during the incubation was measured by radioimmunoassay. The oral activity was confirmed in hypertensive patients (Gradman et al. 2005).

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A.1.1.16**PAF Binding Assay****PRINCIPLE AND RATIONALE**

Injection of platelet activating (PAF) factor induces a wide range of potent and specific effects on target cells, including aggregation of platelets and shock symptoms like systemic hypotension, pulmonary hypertension, increased vascular permeability, neutropenia and thrombocytopenia. Inhalation of PAF causes immediate bronchoconstriction followed by inflammation of the airways (further information see Sect. B.1.9).

Hikiji et al. (2004) showed in PAF-deficient mice that the absence of platelet-activating factor receptor protects mice from osteoporosis following ovariectomy, a model of postmenopausal osteoporosis.

PAF is also implicated in estrogen-induced angiogenesis via nuclear factor- κ B activation (Seo et al. 2004) and in delaying corneal wound healing (Bazan 2005).

The PAF receptor belongs to the superfamily of G protein-coupled receptors (Chao and Olson 1993, Izumi and Shimizu 1995). Cloning studies have indicated a single human PAF receptor gene containing an

intron at the 5' flanking region, providing alternative sequences (Ishi and Shimizu 2000).

The following procedure is used to detect compounds that inhibit binding of ^3H -PAF (platelet activating factor) in rabbit platelets (PAF receptor).

PROCEDURE

Crude rabbit platelets are incubated in plastic tubes for 15 min at 25°C in a buffer solution (0.54 g/l KH_2PO_4 , 0.6 g/l Na_2HPO_4 , 5.8 g/l NaCl, 1.0 g/l BSA, pH 7.1) with 1 nM synthetic ^3H -labeled PAF (1-*O*-[1,2- $^3\text{H}_2$]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) and various concentrations of test compound. Non-specific binding is determined in the presence of 10 μM CV 3988. Bound ligand is separated from the incubation medium by rapid filtration through Whatman GF/C glass fibre filters. Following rinsing with ice-cold buffer (3 \times 5 ml), the filters are placed in 10 ml scintillation cocktail for radioactivity determination.

EVALUATION

The following parameters are calculated:

- total binding of ^3H -PAF
- non-specific binding in the presence of 10 μM CV 3988
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of the control value

Compounds are first tested at a single high concentration (5000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is constructed using 7 different concentrations of test compound. Binding potency of compounds is expressed either as a “relative binding affinity” (RBA) with respect to the standard compound (CV 3988) which is tested in parallel or as an IC_{50} .

$$\text{RBA} = \frac{IC_{50} \text{ standard compound}}{IC_{50} \text{ compound}} \times 100\%$$

Standard data:

- CV 3988 IC_{50} : 276 nM \pm 24 ($n = 20$)

MODIFICATIONS OF THE METHOD

Several authors (Casals-Stenzel et al. 1987; Dent et al. 1989a, b; Ring et al. 1992; Ukena et al. 1988) used the specific platelet activating factor receptor antagonist [^3H]WEB-2086 or [^3H]Apafant to identify and

characterize the PAF-receptors expressed on the cell surface of platelets, macrophages, and eosinophils.

Balsa et al. (1996) characterized [³H]Apafant binding to the PAF receptor on rabbit platelet membranes and compared a microplate filtration system with the standard method.

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A.1.1.17 Endothelin

A.1.1.17.1

General Considerations

Endothelin is an endothelium-derived peptide family consisting of three peptides (ET-1, ET-2, and ET-3) with very potent and long-lasting vasoconstrictive activity (Yanagisawa et al. 1988a, b; King et al. 1989; Miller et al. 1989; Yanagisawa and Masaki 1989; Inoue et al. 1989; Shinmi et al. 1989; Vanhoutte et al. 1992; Davenport 2002; Masaki 2004).

ET-1 is processed from prepro ET-1, pro-ET-1 to big ET-1, which is converted to ET-1 by the endothelin-converting-enzyme (ECE).

Subtypes of endothelin receptors have been described (Takayanagi et al. 1991; Miyazaki et al. 1992).

Molecular characterization of the ET_A and ET_B receptors was reported by Miyazaki et al. (1992), Sakurai et al. (1992).

In addition, the existence of a third type, ET_C, was found in *Xenopus laevis* (Karne et al. 1993).

The comparison of recombinant endothelin receptors shows different affinity rank orders to the three endothelins (Masaki et al. 1994).

Grant et al. (1997) reported the *in vitro* expression of endothelin-1 (ET-1) and the ET_A and ET_B ET receptors by prostatic epithelium and stroma.

The ET peptides not only elicit potent and long-lasting contractions of isolated strips of various blood vessels *in vitro* but also increase blood pressure *in vivo* suggesting that this peptide family may be involved in the pathogenesis of cardiovascular diseases (Simonson and Dunn 1990; Masaki 1991; Doherty 1992; Goto et al. 1996; Gray and Webb 1996; Douglas and Ohlstein 1997). Sarafotoxin S6c, originally isolated from snake venom, is an agonist which distinguishes between endothelin subtypes (Williams et al. 1991).

Endothelin is degraded by vascular smooth muscle cells (Bernmek et al. 1996).

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A.1.1.17.2**Evaluation of Endothelin Activity****PURPOSE AND RATIONALE**

Most investigators used isolated arteries to evaluate the activity of endothelins and derivatives. Rodman et al. (1989) compared the potency and efficacy of porcine and rat endothelin in rat aortic and pulmonary rings.

PROCEDURE

Arterial rings are obtained from male Sprague Dawley rats weighing from 300–400 g. Rats are anesthetized with 50 mg/kg i.p. pentobarbital, the chest is opened, 100 units heparin sulfate are injected into the right ventricle, and the rats are exsanguinated. Rings are then isolated from either the descending thoracic aorta or the right main pulmonary artery, cleaned of adventitia, and suspended from Grass FT03 force-displacement transducers in muscle baths containing 10 ml of physiologic salt solution of the following composition ($\times 10^{-3}$ M): CaCl₂ 1.80, MgSO₄ 0.83, KCl 5.3.6, NaCl 116.34, NaH₂PO₄ 0.40, D-glucose 5.50, and NaHCO₃ 10.04. The solution is maintained at 37°C and bubbled with 21% O₂ and 5% CO₂. Endothelium-denuded rings are prepared by gently rubbing the intima with a roughened steel rod. Denudation is confirmed by the absence of relaxation to 10⁻⁵ M acetylcholine in rings precontracted with 10⁻⁷ M norepinephrine. Resting force is adjusted to the optimum resting tension of 0.75 g for pulmonary artery rings and 1.0 g for aortic rings. Maximum contraction to 8 $\times 10^{-2}$ M KCl is determined and subsequent responses to endothelin are expressed as a percentage of maximum KCl contraction for determination of maximum effectiveness or as a percentage of maximum endothelin contraction for determination of potency.

EVALUATION

Concentration-response curves are compared using the method of Carpenter (1986). Data are expressed as means \pm SEM and statistical comparisons are performed using Student's *t*-test, with $P < 0.05$ considered significant.

MODIFICATIONS OF THE METHOD

Lembeck et al. (1989) studied the effects of endothelin on the cardiovascular system and on smooth muscle preparations in different species.

Reynolds and Mok (1990) studied the role of thromboxane A₂/prostaglandin H₂ receptor in the vasoconstrictor response of **rat aorta** to endothelin.

Pang et al. (1990) studied the cellular mechanisms of action of endothelin in **isolated canine coronary arteries**.

Lüscher et al. (1992) used **perfused and pressurized mesenteric resistance arteries of rats and human internal mammary arteries** to study the interaction between endothelin and endothelium-derived relaxing factors.

Michel et al. (2003) studied the endothelin system in various animal models of pulmonary hypertension.

Advenier et al. (1990) studied the contractile activity of three endothelins (ET-1, ET-2 and ET-3) on the **human isolated bronchus**.

Wallace et al. (1989) compared the effects of endothelin-1 and endothelin-3 on the **rat stomach**.

Aldosterone secretion in cultured calf zona glomerulosa cells was stimulated by ET-1 and sarafotoxin S6b to a similar degree, but less than by angiotensin II (Gomez-Sanchez 1990).

Brock and Danthuluri (1992) used **cultured vascular smooth muscle cells** to study the cellular actions of endothelin.

Pigment dispersion in cultured dermal melanophores from *Xenopus laevis* was used as indicator of ET_C receptor mediated responses (Karne et al. 1993).

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A.1.1.17.3

Endothelin Receptor Antagonism in Vitro

PURPOSE AND RATIONALE

Competitive endothelin antagonists are of therapeutic interest (Ihara et al. 1991; Fujimoto et al. 1992; Fukuroda et al. 1992; Urade et al. 1992; Breu et al. 1993; Mihara and Fujimoto 1993; Sogabe et al. 1993; Warner 1994; Opgenorth 1995; Brunner 1998).

A sensitive sandwich-enzyme immunoassay for human endothelin has been established by Suzuki et al. (1989).

PROCEDURE

The ventricles of rat hearts are minced with scissors and homogenized in 7 vol of ice-cold 20 mM NaHCO₃ containing 0.1 mM PMSF (Phenylmethylsulfonyl fluoride), pH 7.4, with a Polytron homogenizer (Brinkman Instruments Inc., Westberg, NY). The homogenates are centrifuged at 1000 *g* for 10 min, and then the pellet discarded. The supernatant is centrifuged at 30,000 *g* for 30 min. The pellet is washed once and resuspended in Tris buffer (50 mM, pH 7.4 at 25°C) containing 0.1 mM PMSF, and stored at –80°C until use.

For binding studies (Gu et al. 1989) cardiac membranes (0.21 mg/ml as protein) are incubated

with 25 pM [125 I]ET-1 or [125 I]ET-3 (New England Nuclear) in a final assay volume of 0.1 ml in borosilicated glass tubes, containing 50 mM Tris-HCl, 0.1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 250 μ g/ml bacitracin, and 10 μ g/ml soybean trypsin inhibitor (pH 7.4). Binding is performed for 60 min at 37°C. The binding reaction is terminated by the addition of 2.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), followed by a rapid filtration through a Whatman GF/C glass fibre filter (pre-soaked in 1% polyethyleneimine) under reduced pressure. The filters are then quickly washed 4 times with 2.5 ml of the buffer. Radioactivity retained on the filter is counted.

EVALUATION

Non-specific binding is defined in the presence of ET-1. Specific binding is the difference between total and non-specific binding. K_i -values and Scatchard plots are calculated.

MODIFICATIONS OF THE METHOD

The nomenclature of endothelin receptors has been reviewed by Alexander et al. (2001).

Cain et al. (1991) described an endothelin-1 receptor binding assay for high throughput chemical screening using the clonal cell line A10 of smooth muscle cells, derived from embryonic rat thoracic aorta.

Functional endothelin/sarafotoxin receptors were described in rat heart myocytes (Galron et al. 1989) and in the rat uterus (Bouso-Mittler 1989).

Mihara and Fujimoto (1993) cultured rat aortic smooth muscle A7r5 cells expressing ET_A receptors (Takuwa et al. 1990) and human Girardi heart cells expressing ET_B receptors (Mihara and Fujimoto 1992). Receptor specificity could be demonstrated.

Mihara et al. (1994) characterized the nonpeptide endothelin receptor antagonist 97-139, both *in vitro* (rat aortic smooth muscle cells and Girardi heart cells) and *in vivo* (ET1-antagonism in pithed rats) and compared it with another endothelin receptor antagonist (BQ-123). Discrepancies between *in vitro* and *in vivo* data were explained by different plasma binding.

Aramori et al. (1993) studied the receptor-binding properties and the antagonistic activities of an endothelin antagonist in transfected Chinese ovary hamster cells permanently expressing the two ET receptor subtypes (ET_A and ET_B).

De Juan et al. (1993) characterized an endothelin receptor subtype B in the retina of rats.

Clozel et al. (1994) performed binding assay on cells or membranes from baculovirus infected insect

cells that expressed recombinant ET_A or ET_B receptor, CHO cells that expressed recombinant ET_A or ET_B receptor, cultured human vascular smooth muscle cells from umbilical veins, rat mesangial cells (for ET_A), microsomal membranes from human placenta and from porcine cerebellum (for ET_{B1}) and from porcine trachea (for ET_{B2}, using BQ-3020 or sarafotoxin S6C as ligand).

Williams et al. (1995) used CHO cells expressing cloned ET_A or ET_B receptors directly in binding and functional assays without preparing membranes from them.

Reynolds et al. (1995) used CHO-K1 cells expressing recombinant human ET_B receptor, Ltk⁻ cells expressing human ET_A receptor and rabbit renal artery vascular smooth muscle cells expressing rabbit ET_A receptor for evaluation of an ET_A receptor antagonist.

Rat or bovine cerebella were used for differentiation of receptor subtypes (Williams et al. 1991).

Peter and Davenport (1995) proposed a selective ligand for ET_A receptors.

Ihara et al. (1992), Watakabe et al. (1992) described radioligands for endothelin (ET_B) receptors.

Vigne et al. (1996) described the properties of an endothelin-3-sensitive Eta-like endothelin receptor in brain capillary endothelial cells.

The human type-B endothelin receptor was cloned from human lung poly A+RNA and expressed in CHO cells by Chiou et al. (1997). Dissociation characteristics of endothelin receptor agonists and antagonists were determined.

Stables et al. (1997) described a bioluminescent assay for agonist activity at G-protein-coupled receptors, such as the endothelin ET_A receptor. Transient expression of apoaequorin in CHO cells and reconstitution with the cofactor coelenterazine resulted in a large, concentration dependent agonist-mediated luminescent response following cotransfection with the endothelin ET_A, angiotensin AT_{II}, TRH and neurokinin NK₁ receptors, all of which interact predominantly with the G α_q -like phosphoinositidase-linked G-proteins.

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Endothelin Receptor Antagonism in Vivo

PURPOSE AND RATIONALE

Various pharmacological models have been used for the characterization of endothelins and endothelin antagonists, such as the isolated porcine coronary artery. (Hickey et al. 1985; Yanagisawa et al. 1988, 1989; Inoue et al. 1989; Kimura et al. 1989; Ihara et al. 1991; Fukuroda et al. 1991).

Since the smooth musculature is considered to contain mainly ET_A receptors the preparation is used to test ET_A antagonists.

PROCEDURE

Left anterior descending coronary arteries are isolated from fresh porcine hearts. Connective tissues and adherent fat are removed. For removal of vascular endothelium, the intimal surface of spiral strips is rubbed gently with filter paper. The endothelium-denuded arteries are cut into spiral strips about 10 mm long and 1 mm wide. Each strip is suspended in an organ bath containing Krebs-Henseleit solution bubbled with 95% O₂/5% CO₂ at 37°C. After equilibration, reference contraction is isometrically obtained with 50 mM KCl. Concentration-response curves for ET-1 are obtained by cumulative additions of ET-1. Antagonists are added 20 min before the cumulative additions of ET-1.

EVALUATION

The pA₂ values and slopes are obtained by analysis of Schild plots.

MODIFICATIONS OF THE METHOD

Opgenorth et al. (1996) characterized an orally active and highly potent ET_A-selective receptor antagonist by *in vitro* and *in vivo* methods.

Calo et al. (1996) investigated three **rabbit vessels, the carotid, the pulmonary artery, and the jugular vein** to identify vascular monoreceptor systems, either ET(A) or ET(B), for structure-activity studies of endothelins and their antagonists.

Vedernikov et al. (1993) used **rings of the left circumflex coronary artery from dogs** which were denuded of endothelium and exposed to anoxic periods. August et al. (1989), Urade et al. (1992) used **rat aortic smooth muscle denuded of the epithelium** and Sogabe et al. (1993) **spirally cut strips of rabbit aorta**.

Williams et al. (1995) used **rat aorta, rabbit iliac and pulmonary artery** for contractile assays, and **anesthetized ferrets and conscious normotensive dogs** as *in vivo* models to characterize a nonpeptidyl endothelin antagonist.

Itoh et al. (1993) studied the preventive effect of an ET_A receptor antagonist on **experimental cerebral vasospasm in dogs** using a two-hemorrhage model of subarachnoid hemorrhage. Clozel et al. (1993) performed similar experiments in rats.

The **vasodilating effect in the isolated perfused rat mesentery** which is found after infusion of rat endothelin (Warner et al. 1989) and after the selective ET_B receptor agonist sarafotoxin S6c (Williams et al. 1991) can be antagonized by an endothelin receptor antagonist (Clozel et al. 1993).

Ercan et al. (1996) found an increase of digoxin-induced ectopic ventricular complexes by endothelin peptides in **isolated guinea pig hearts**, which could be antagonized by an endothelin-A receptor antagonist.

The **endothelin-induced sustained increase of blood pressure** in anesthetized rats was studied by Yanagisawa et al. (1988), Inoue et al. (1989), Ihara et al. (1991). Intravenous bolus injection of endothelin causes a biphasic blood pressure response: a transient decrease, probably mediated from the release of vasodilator mediators (prostacyclin and EDRF), and a sustained increase (Rubanyi and Bothelho 1991).

Nishikibe et al. (1993) examined the **antihypertensive effect** of an endothelin antagonist in a genetic hypertensive model (**stroke-prone spontaneously hypertensive rats**).

Watanabe et al. (1995) characterized the pharmacological profile of a non-selective endothelin receptor antagonist and studied the **inhibition of myocardial infarct size** in rats.

The contractile activity of the **isolated guinea pig trachea without epithelium** and of the guinea pig longitudinal muscle was used by Urade et al. (1992) for determination of **ET_B receptor** mediated responses.

Spinella et al. (1991) assessed bioactivity of a specific endothelin-1 antagonist in an **isolated perfused guinea pig lung** preparation in which pulmonary artery pressure was monitored.

Gosselin et al. (2002) demonstrated the effects of a selective ET_A-receptor antagonist in murine models of allergic asthma.

Tabrizchi and Ford (2003) studied the haemodynamic effects of the endothelin receptor antagonist tezosentan in anaesthetized rats treated with tumor necrosis factor-alpha.

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A.1.1.17.5

Quantitative Autoradiographic Localization of Endothelin-1 Receptor

PURPOSE AND RATIONALE

The endothelin-1 (ET-1) receptor can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized *in vitro* autoradiography (Kohzuki et al. 1996)

PROCEDURE

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see A.3.2.2). After various time intervals (1–8 months) the animals are decapitated, the hearts rapidly removed, and snap-frozen in isopentane at –40°C. Frozen section (20 μm) are cut in a cryostat at –20°C. The sections are thaw-mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4°C and then stored at –80°C.

Quantitative autoradiography

Radioligand: Endothelin-1 is iodinated with ¹²⁵Iodine using Iodogen (Pierce Chemical Co, IL, USA)

¹²⁵I-ET-1 binding: The sections are preincubated for 15 min at 20°C in 20 mmol/L Hepes buffer, pH 7.4, containing 135 mmol/L NaCl, 2 mmol/L CaCl₂, 0.2% BSA, and 0.01% bacitracin. The sections are then incubated with 11.1 KBq/ml ¹²⁵I-ET-1 in the same buffer for 60 min at 20°C. Nonspecific binding is determined in the presence of 10^{–6} mol/L ET-1. Binding isotherms are determined using a set of serial sections incubated with 10^{–12} to 10^{–6} mol/L unlabelled ET-1 for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h at room temperature. After exposure, the sections are fixed in formaldehyde and stained with hematoxylin and eosin. The optical density of the X-ray films is

quantified using an imaging device controlled by a personal computer.

EVALUATION

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm² with reference standards maintained through the procedure. The apparent binding site concentration (B_{\max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle and the non-infarcted area in the left ventricle are estimated by an iterative non-linear model-fitting computer program LIGAND (Munson and Rodbard 1980).

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A.1.1.17.6

Inhibition of Endothelin Converting Enzyme

PURPOSE AND RATIONALE

Endothelin converting enzyme inhibitors suppress the biosynthesis of endothelin are therefore potential antihypertensive drugs (De Lombaert et al. 1994; Trapani et al. 1995; Morita et al. 1994; Bihovsky et al. 1995; Claing et al. 1995; Descombes et al. 1995; Chackalamannil et al. 1996; Jeng 1997; Jeng and De Lombaert 1997; Brunner 1998).

Purification of rat and porcine endothelin converting enzyme (ECE) was reported by Ohmaka et al. (1993), Takahashi et al. (1993). Molecular cloning and characterization of the enzyme ECE-1 was performed from rat (Shimada et al. 1994), bovine (Ikura et al. 1994; Schmidt et al. 1994; Xu et al. 1994), and human tissue (Schmidt et al. 1994; Shimada et al. 1995; Yorimitsu et al. 1995).

A second enzyme, termed ECE-2, was cloned (Emoto and Yanagisawa 1995).

Walkden and Turner (1995) described the expression of endothelin converting enzyme and related membrane peptidases, e. g., the endopeptidase E-24.11, in the human endothelial cell line EA.hy926.

IN VITRO ASSAY

A rapid and selective *in vitro* assay for endothelin-converting enzyme was described by Fawzi et al. (1994). The assay is based on the quantitative determination of [¹²⁵I]endothelin-1 released from (3-[¹²⁵I]iodotyrosyl¹³)big endothelin-1 by binding to the membrane bound endothelin receptor.

PROCEDURE

For the **preparation of lung membranes**, frozen guinea pig lungs are weighed and homogenized in 10 times gram tissue weight of solution A (50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 2 mM EDTA) using a Polytron tissue homogenizer. Homogenization is repeated 4 times with 5- to 8-min intervals between homogenization. Homogenates are spun for 30 min at 2000 g. Supernatants containing membranes are carefully decanted and saved. Pellets are rehomogenized in solution A and homogenates are spun at 2000 g for 30 min. Supernatants are removed, mixed with supernatants from the first spin and spun at 100,000 g for 60 min. Pellets containing membranes are suspended in solution B (10 mM Tris-HCl, pH 7.4, and 0.125 M sucrose) using a Dounce homogenizer. Samples are divided into 1-ml fractions, rapidly frozen in a dry ice-methanol bath, and stored at –80°C.

Rat liver membranes are prepared with the same method and further purified over a sucrose step gradient. The membranes are suspended in solution C (10 mM Tris-HCl, pH 7.4) containing 44% sucrose at a protein concentration of 2 mg/ml. Samples of 25 ml are placed in ultraclear centrifuge tubes for the Beckman SW 28 rotor, overlaid with 10 ml solution C containing 42.3% sucrose, and spun for 2 h at 27,000 rpm (100,000 g). Top layers containing membrane are collected and diluted with solution C to obtain an 8% sucrose concentration. Samples are spun in a 45 Ti rotor (100,000 g) for 1 h. Supernatants are discarded. Pellets containing membrane are suspended in solution B, divided into 1-ml samples, rapidly frozen in dry ice-methanol bath, and stored at –80°C.

For the **endothelin (ET) binding assay**, membrane preparations are incubated with selected concentrations of [¹²⁵I]endothelin-1 (final reaction volume = 500 µl) in a solution D containing 60 mM Tris-

HCl, pH 7.4, 150 mM NaCl and 6 mg/ml BSA for 90 min at 37°C. Reactions are terminated by the addition of 4 ml of solution E containing 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl at 4°C followed by rapid filtration on Whatman GF/B glass microfiber filters. Filters are presoaked for 1 h at 4°C in a solution containing 50 mM Tris-HCl, pH 7.4, 10 mg/ml BSA and 0.1% sodium azide. Test tubes and filters are washed four times with 4 ml of solution E at 4°C, and radioactivity retained on the filters is counted in a gamma counter. Nonspecific binding is determined in the presence of 1 μ M unlabeled ET-1 in the reaction mixture.

For the **endothelin converting enzyme assay**, samples containing 10 μ g of protein are incubated in a solution containing 50 mM Tris-HCl, pH 7.0, 100 mM NaCl and 5 mg/ml BSA in a final volume of 100 μ l. Conversion reactions are initiated by addition of [¹²⁵I]big endothelin-1 to obtain a final concentration of 500 pM. Samples are incubated for 2 h at 37°C. To measure [¹²⁵I]endothelin-1 released from [¹²⁵I]big endothelin-1 conversion, 50 μ g of purified rat liver membranes (as a source of ET receptors) is added to the reaction mixture, and reaction volume is adjusted to 500 μ l and solution composition is adjusted to that of solution D of the endothelin binding assay. Following a 90-min incubation at 37°C to reach equilibrium in binding, reactions are terminated by addition of a solution E at 4°C followed by rapid filtration on Whatman GF/B glass microfiber filters. Nonspecific binding is determined in the presence of 1 μ M unlabeled ET-1 in the reaction mixture. Specific ET-1 binding is used as an index of endothelin converting enzyme activity.

To test the effect of endothelin converting enzyme inhibitors, endothelin converting enzyme assays are carried out in the presence of desired concentrations of the compounds.

EVALUATION

Endothelin converting enzyme activity in the presence of compounds is expressed as a percentage of control endothelin converting enzyme activity in the membrane preparation which is determined simultaneously. The concentration of compounds producing a 50% inhibition of endothelin converting enzyme activity (*IC*₅₀ values) is determined from a plot of the percentage of control endothelin converting enzyme activity versus log concentration of compounds.

IN VIVO ASSAY

Procedure

Male Sprague Dawley rats weighing 300–400 g are anesthetized with ether, spinalized and placed under

artificial respiration. The vagus nerves are cut and the carotid arteries ligated. A catheter is placed in one of the carotid arteries to allow measurement of arterial blood pressure. The second catheter is placed into the penile vein to allow infusion or injection of drugs. After stabilization, the animals receive a first injection of either ET-1, big ET-1, norepinephrine, angiotensin I or AT II. The pressor responses are recorded and after return to the baseline, a second injection of the agonist is given either in the presence or the absence of the inhibitor.

EVALUATION

Data are calculated as mean \pm SEM. Student's *t*-test for paired and unpaired observations is used to analyze the results.

MODIFICATIONS OF THE METHOD

Little et al. (1994) developed a two-step protocol for high-throughput assays of endothelin converting enzyme activity. Human umbilical vein and human aorta endothelial cells were found to preferentially convert the big endothelin-1 isopeptide through a membrane-bound, thiorphan-insensitive, and phosphoramidon-sensitive zinc metalloendopeptidase. Endothelins are quantified by a separate step using either enzyme immunoassays or radioceptor assays in 96-well formats. The method can be used to either characterize ECE from different tissues or screen for inhibitors of a specific ECE activity.

McMahon et al. (1993) tested the effects of endothelin converting enzyme inhibitors and endothelin receptor subtype A antagonists on blood pressure in spontaneously and renal hypertensive rats.

Changes of vascular resistance in isolated perfused kidneys were used by Descombes et al. (1995) to characterize a selective inhibitor of big ET-1 responses. The studies were performed on kidneys taken from adult male Wistar rats (300–400 g). The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and the left kidney was prepared for infusion with Tyrode solution. The changes in renal vascular resistance were recorded as changes in perfusion pressure monitored at constant flow (6 ml/min). After stabilization, a bolus injection of ET-1 or big ET-1 was administered and the resulting pressure responses were recorded. On return to baseline levels, a second injection of the endothelins was given either under control conditions or in presence of the putative enzyme inhibitor.

Because increasing evidence implicates that endothelin plays a role in the pathophysiology of cerebral insults, Kwan et al. (1997) studied the prevention

and reversal of cerebral vasospasm in an experimental model of subarachnoid hemorrhage. Three ml of arterial blood was withdrawn from the ear artery of rabbits and injected into the cisterna magna under anesthesia. Drugs were administered either before or 24 h after this procedure. Forty-eight hours later, the animals were anesthetized again and perfusion fixation was performed with Hank's balanced salt solution followed by a mixture with 2% paraformaldehyde and 2.5% glutaraldehyde. Cross sections of the basilar arteries were analyzed by computer-assisted morphometry.

A review on the knowledge of molecular pharmacology of endothelin converting enzymes was given by Turner and Murphy (1996).

Johnson and Ahn (2000) developed an internally quenched fluorescent substrate selective for endothelin-converting enzyme-1.

Luciani et al. (2001) described highly sensitive and selective fluorescence assays for rapid screening of endothelin-converting enzyme inhibitors.

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A.1.1.18

Nitric Oxide

A.1.1.18.1

General Considerations on Nitric Oxide

PURPOSE AND RATIONALE

The endothelium releases a labile, diffusible vasorelaxing substance that has been termed endothelium-derived relaxing factor = EDRF endothelium-derived relaxing factor. (Furchgott and Zawadzki 1980). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor (Palmer et al. 1987; Vanhoutte 1999).

Nitric oxide plays a role in a wide range of physiological processes including regulation of blood flow and arterial pressure via endothelium-dependent relaxation of blood vessels (Rees et al. 1989; Moncada et al. 1991; Umans and Levi 1995; Huraux et al. 1999; McIntyre et al. 1999; Zanzinger 1999; Hropot et al. 2003), ischemia/reperfusion injury (Gao et al. 2002; Schulz et al. 2004), peripheral nitregic transmission at smooth muscle (Rand and Li 1995), intracellular communication in the CNS with activation of guanylyl cyclase in target neurons (Southam and Garthwaite 1993), experimental stroke (Willmot et al. 2005), learning and memory (Susswein et al. 2004), in neurogenic inflammation (Kajekar et al. 1995), in the regulation of leukocyte recruitment (Hickey 2001), and macrophage defense mechanisms following exposure to bacterial products (Förstermann et al. 1992; Förstermann and Kleinert 1995; Knowles and Moncada 1994). Fiorucci et al. (2002) discussed the effects of nitric oxide-releasing NSAIDs.

NO-donor drugs, such as sodium nitrite, sodium nitroprusside, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), 3-morpholino-sydnominine (SIN-1) are used as vasodilators (Schör et al. 1989; Megson 2000). N^G -Nitro-L-arginine was described as an antagonist of endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release (Moore et al. 1990; Lamontagne et al. 1991). Ribero et al. (1992) proposed inhibition of nitric oxide synthesis by long-term treatment of rats with nitro-L-arginine as a new model of arterial hypertension.

Excessive production of NO damages DNA and activates poly(ADP-ribose)polymerase (PARP) (Pieper

et al. 1999). In cases of massive NO production, neurons enter the PARP-suicide pathway. NO damages DNA via to major pathways: the first involves nitrosation of primary or secondary amines and nucleic acid bases, whereas the second involves the combination of NO with superoxide to form peroxynitrite (Szabó et al. 1996, 1997). The most likely reactive oxidant intermediate responsible for DNA breakage is peroxynitrous acid which rapidly oxidizes sulphhydryl groups, and also nitrates and hydroxylates aromatic compounds including tyrosine, tryptophan, and guanosine (Halliwell 1997). Downstream DNA damage that follows excessive NO production results in significant activation of poly(ADP-ribose)polymerase which leads to rapid energy depletion and cell death (Feihl et al. 2001).

Davis et al. (2001) reviewed the non-3',5'-cyclic-guanosine-monophosphate-mediated effects of NO including modifications of proteins, lipids, and nucleic acids.

Andreadis et al. (2003) described oxidative and nitrosative events in asthma.

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A.1.1.18.2

Bioassay of EDRF Release

PURPOSE AND RATIONALE

EDRF release from arterial endothelium can be studied by a sandwich technique using donor tissue with intact endothelium facing with its intimal side the intimal side of a detector tissue.

PROCEDURE

Rabbits are subjected to various kinds of treatment, e. g., atherogenic diet or drug treatment for prevention of arteriosclerosis. Aorta segments, about 2 cm in length, are prepared, cut open along their longitudinal axis and pinned to a tissue suspender without damaging the endothelium. These segments serve as donor tissue for EDRF. Circumferential aorta strips from the abdominal aorta of untreated control rabbits are de-endothelialized by gently blotting their luminal surfaces on wet filter paper. These denuded abdominal aorta strips are pinned opposite the donor segments (intimal surface facing intimal surface) and function as detector for lumenally released EDRF. Each sandwich preparation is suspended in a 40-ml organ bath, filled with oxygenated Krebs-Ringer buffer at 37°C containing 10 mM indomethacin. After connecting the detector strip to a force transducer, the angle between the detector strip and the donor segment is minimized and the distance between donor and detector tissue standardized. After one hour stabilization, the strips are brought to their optimum length-tension relationship by repeated exposure to 80 mM KCl. When a stable contractile response is established, the strips are precontracted with phenylephrine to 80–100% of their KCl-induced contraction. After stabilization of plateau phase, cumulative doses of acetylcholine (0.01–10 mM) are added to induce EDRF release from donor tissues.

EVALUATION

Relaxations of the detector strip induced by EDRF release from treated donor rabbit aortas are compared with aortas from control rabbits.

MODIFICATIONS OF THE METHOD

An other bioassay for measuring function of cultured endothelial cells using a computer system for the acquisition and analysis of vascular contractility has been published by Winn et al. (1992).

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A.1.1.18.3

Isolated Arteries with and Without Endothelium

PURPOSE AND RATIONALE

Endothelial cells are able to synthesize and release potent vasoconstrictive agents, such as endothelin and angiotensin as well as vasodilating agents, such as EDRF. In isolated arterial segments the endothelial surface can be functionally destroyed allowing a differentiation between a direct action of drugs on the smooth muscle cells and an indirect effect via the endothelium. Isolated rings of rabbit or rat aorta are useful models to study the effects of endothelium derived factors such as EDRF or endothelins and their antagonists (Linz et al. 1986; Tracey et al. 1990; Fujimoto et al. 1992; Fukuroda et al. 1992; Wiemer et al. 1992). A survey of the history and on techniques leading to the discovery of endothelium-dependent relaxation was given by Furchgott (1993).

PROCEDURE

The descending thoracic aorta from rabbits of either sex (weighing 2.5–3.5 kg) is excised and dissected free from connective tissue. Care is taken to avoid damage of the endothelium. The aorta is divided into 2 mm wide rings and cut off in small strips. From some strips, the endothelium is removed by gently rubbing the intimal surfaces between the fingers for approximately 30 s. The strips are suspended in a 25 ml organ bath containing Krebs-bicarbonate solution at 37°C being gassed continuously with 5% CO₂/95% O₂. Contractions of the strips are recorded isometrically with a load of 2 g on the tissues. After an equilibration period of 2 h a stable baseline tone is reached.

To study the vasodilating effects of a compound, the strips are contracted with norepinephrine (10⁻⁸ M),

or angiotensin II (10⁻⁷ M), or potassium chloride (20 mM). When a stable contraction plateau has been reached, the vasodilating agent is added in various concentrations. In these concentrations, norepinephrine, angiotensin II and KCl evoke a response of 60–80% of maximal contraction in intact rings of rabbit aorta. Rings without endothelium exhibit a response which is significantly enhanced in comparison with the response of the intact preparation after norepinephrine and angiotensin II precontraction.

To indicate the functional removal of the endothelium, the responsiveness of each preparation is tested with the known endothelium-dependent dilator, acetylcholine. In endothelium-intact rings, acetylcholine relaxes contractions induced by norepinephrine or angiotensin II. In precontracted rings devoid of endothelium, acetylcholine does not show any relaxing effect or causes contractions by itself at higher concentrations (Furchgott and Zawadzki 1980). As an example, atriopeptin III causes a similar concentration-dependent relaxation of all precontracted preparations with intact and with functionally destroyed endothelium indicating a direct effect on the smooth muscle cells. The relaxation is accompanied by an increase of cGMP.

EVALUATION

Statistical analyses are performed by regression analysis of dose response curves to determine EC₅₀ values. Data are given as means ± standard deviation.

CRITICAL ASSESSMENT OF THE METHOD

The isolated aortic ring of rabbits with and without functionally intact endothelium is a useful tool to differentiate direct effects on the arterial smooth musculature from effects mediated by the endothelium.

MODIFICATIONS OF THE METHOD

Fujimoto et al. (1992) used the thoracic aorta from rats to study the effects between endothelin and an endothelin receptor antagonist. In transverse strips from **rat** thoracic aorta, 2 mm wide and 4–5 mm long, the endothelium was removed by gently rubbing the interior surface of the aorta. Concentration-response curves of contractions after ET-1 in the presence and the absence of the inhibitor were compared.

Pellisier et al. (1992) perfused the isolated mesenteric vascular bed of the **rat** with Tyrode solution and measured the perfusion pressure after injection of graded doses of norepinephrine and the dose-dependent relaxation due to acetylcholine in the vascular bed precontracted by norepinephrine infusion. In order to destroy the endothelial layer, the perfusate was

changed to a hypotonic Tyrode solution containing all of the constituents present in normal Tyrode solution but in one-tenth of the concentration resulting in disruption of more than 95% of the endothelial cells. The effect of norepinephrine was enhanced, whereas the effect of acetylcholine was abolished.

Legan and Sisson (1990) described a method to denude **rat** aortic endothelium *in vitro* with saponin.

Bohn and Schönafinger (1989) used helical strips of pulmonary arteries of **guinea pigs** in which the endothelium has been removed for biological detection of NO.

Fukuroda et al. (1992) used spiral strips from **porcine** coronary artery and vein and from intrapulmonary artery and vein removing the intimal surface by lightly rubbing with wet filter paper. Concentration-contraction curves for ET-1 and ET-3 were obtained with and without an endothelin antagonist.

Hayashi et al. (1988) described functional and anatomical recovery of endothelium after balloon denudation of the left circumflex coronary artery in **dogs**.

Endothelial denudation of the left circumflex coronary artery was used by Chu and Cobb (1987) to study the vasoactive effects of serotonin on proximal coronary arteries in awake **dogs**.

Experiments in isolated rings of the left circumflex or left anterior descending coronary artery of **dogs** with and without endothelium were performed by Desta et al. (1995).

Terrón (1996) analyzed the effects of 5-HT₁-receptor antagonists on 5-HT and sumatriptan induced isometric contractions in endothelium denuded segments of **canine** coronary arteries.

Ren et al. (1993) isolated coronary arteries from Japanese **monkeys** (*Macaca fuscata*) with and without endothelium to study muscarinic receptor subtypes mediating vasodilatation and vasoconstriction.

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A.1.1.18.4

Nitric Oxide Formation by Cultured Endothelial Cells

PURPOSE AND RATIONALE

Endothelial cells are able to synthesize and to release not only potent vasoconstrictor peptides such as an-

giotensin and endothelin but also potent dilators such as nitric oxide (NO), ATP, substance P, and bradykinin.

NO-formation can be assessed by determination of intracellular cyclic GMP in cultured endothelial cells, whereas release of NO from these cells can be measured by the stimulatory effect of NO on the activity of soluble guanylyl cyclase (Lückhoff et al. 1988; Wiemer et al. 1991; Linz et al. 1992; Bogle et al. 1992; review by Moncada et al. 1991).

PROCEDURE

Endothelial cell culture

Bovine or porcine aorta is obtained from local slaughter houses. Endothelial cells are isolated by digestion with dispase (Lückhoff et al. 1988). The cells are seeded on 6- or 24-well plates (e.g., Nunc Intermed, Wiesbaden, Germany) and grown to confluence. Dulbecco's modified Eagle's/Ham's F-12 medium containing 20% fetal calf serum is supplemented with penicillin (10 U/ml), streptomycin (10 µg/ml), L-glutamate (1 mM/l), glutathione (5 mg/ml), and L(+)-ascorbic acid (5 mg/ml); (Biotect protection medium).

Measurement of cyclic GMP

Primary cultures of endothelial cells are used. After removal of the culture medium by aspiration, the monolayer is washed twice with 2 ml HEPES-Tyrode's solution (37°C). Thereafter, the cells are preincubated for 15 min at 37°C with 3-isobutyl-1-methylxanthine (IBMX), (10^{-4} M/l). After this time, drugs or solvents are added. After predetermined periods, the incubation medium is quickly removed. The cells are then immediately extracted with 0.6 ml 6% trichloroacetic acid and scraped off with a rubber scraper. The cell suspension is sonicated for 10 s before being centrifuged for 5 min at 4000 g. The supernatants are extracted with four volumes of water saturated diethylether, and the samples frozen (-20°C) until analysis. The protein contents of the samples are measured according to Lowry et al. (1951). Cyclic GMP can be determined in the acetylated samples by various methods (Heath et al. 1992), e.g., using a commercially available radioimmunoassay (New England Nuclear). Cyclic GMP content is expressed as picomoles GMP per milligram protein.

Measurement of NO release

Release of NO from endothelial cells is assayed on the basis of the stimulatory effect of NO on the activity of soluble guanylyl cyclase (purified from bovine lung

(Gerzer et al. 1981). The activity of the enzyme is determined in terms of the formation of cyclic [32 P]GMP from α -[32 P]GTP. Reactions are carried out in a reaction mixture containing 30 mM triethanolamine-HCl (pH 7.4), 1 mM reduced glutathione, 4 mM MgCl₂, 1 mM cGMP and 0.1 mg/ml bovine γ -globulin (total volume of 0.18 ml) at 37°C in the presence of α -[32 P]GTP (0.03 mM; 0.2 µCi) and soluble guanylyl cyclase (4 µg). Ten-µl samples are quickly transferred to the reaction mixture. Enzymatic formation of cGMP is allowed to proceed for 60 s and then stopped by the addition of 450 µl zinc acetate (120 mM) and 500 µl sodium carbonate (120 mM). A complete inhibition of cGMP formation can be achieved by preincubation of the monolayers for 30 min with the stereospecific inhibitor of NO synthase, N^G-nitro-L-arginine.

EVALUATION

Time-response curves and dose-response curves after addition of various activators or inhibitors of NO synthase are established. Data are reported as mean values \pm SEM of cGMP (pmol/mg protein) or guanylyl cyclase activity (nmol/mg/min). Statistical evaluation is performed with Student's *t*-test.

MODIFICATIONS OF THE METHOD

The clinical pharmacology of L-arginine has been reviewed by Böger and Bode-Böger (2001).

Isolation of porcine cerebral capillary endothelial cells has been described by Wiemer et al. (1994).

Feelisch and Noack (1987) and Nakazawa et al. (1992) used chemiluminescence techniques for determination of NO.

A method for on-line detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy was described by Mordvintcev et al. (1991). Similar methods were used by Ichimori et al. (1992), Lancaster et al. (1992), Steel-Goodwin et al. (1992).

Hecker et al. (1995) used a cascade superfusion bioassay to characterize a stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells.

Electrochemical microprobes for direct measurement of NO in tissues have been developed (Shibuki 1990; Ishida et al. 1996; Smits and Lefebvre 1997).

Malinski and Taha (1992), Linz et al. (1999) measured nitric oxide release by a porphyrinic-based microsensor with a detection limit of 10 nmol/L. The amperometric signal at a constant potential of 0.67 V was measured with a voltametric analyzer (PAR model 273, Princeton Applied Research) interfaced

with an IBM 80486 computer with data acquisition and software.

Gabriel et al. (1997) developed a method for the detection of intracellular nitric oxide generation in dissociated cerebellar granule cells using dichlorofluorescein diacetate and flow cytometry.

Sumpio et al. (1987) found that cyclic mechanical stress stimulates cultured bovine aortic endothelial cells to proliferate.

Using this method, Rosales et al. (1997) found that exposure of endothelial cells to cyclic strain induces elevations of cytosolic Ca^{2+} concentration through mobilization of intracellular and extracellular pools.

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A.1.1.18.5

Expression of Nitric Oxide Synthase

PURPOSE AND RATIONALE

Properties of various forms of nitric oxide synthase (NOS) have been described by Mayer et al. (1992), Leone et al. (1992), Hevel et al. (1992), Förstermann et al. (1992, 1995), (Salter et al. 1992), Pollock et al. (1992), Schmidt et al. (1992), and Mungrue et al. (2003), among them type I, which is constitutively expressed in neurons; the inducible type II which is found in macrophages and hepatocytes, but also in the brain (Moro et al. 1998), where it may contribute to NO-mediated neurotoxicity; and type III, which is constitutively expressed in endothelial cells (Knowles and Moncada 1994). NOS can be inhibited by several routes, e.g., competition with L-arginine, NADPH, flavin or tetrahydrobiopterin, interaction of the haeme group of NOS, interference with Ca²⁺ availability or calmodulin binding to the enzyme (Fukuto and Chaudhuri 1995). A widely used inhibitor is L-NAME (Vargas et al. 1991). Selective inhibition of constitutive NOS can be achieved by 7-nitroindazole (Moore et al. 1993); of the inducible NOS by aminoguanidine hydrochloride (Griffiths et al. 1993) and by 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (= AMT) (Nakane et al. 1995). Linz et al. (1999) determined NOS in the left cardiac ventricle of hypertensive rats.

PROCEDURE

Tissues are ground at the temperature of liquid nitrogen using a microdismembrator (Braun). The powders are extracted for 1 h on ice with 10 mmol/l Tris-HCl, pH 7.4, containing 1% SDS and protease inhibitors (complete, Boehringer Mannheim). Debris is removed by a 30-min centrifugation at 4°C (>100,000 g). 100 µg of total of the protein extracts are subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Hybond, Amersham). The eNOS protein is detected by use of a specific antibody (monoclonal anti-NOS III, Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). As a secondary antibody, an anti-mouse IgG antibody coupled to alkaline phosphatase is used (Jackson ImmunoResearch Laboratories). Chemifluorescence is analyzed and quantified by scanning with a Fluorimager 595-system (Molecular Dynamics).

EVALUATION

The data are given as mean ± SEM. ANOVA is used followed by Tuckey's test for post-ANOVA multiple pair comparisons.

MODIFICATIONS OF THE METHOD

Linz et al. (1997) measured expression of eNOS in the carotid artery of hypertensive rats by Western blot analysis. Frozen (−70°C) vessels were thawed and extracted with guanidium isothiocyanate/phenol/chloroform (Chomczynski and Sacchi 1987). Crude protein fractions were obtained by alcohol precipitation of the phenol phase. A total of 100 µg of the protein extracts was subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Ponceau staining was performed to verify the quality of the transfer and the equipartition of protein in each lane. eNOS protein was detected with a specific antibody (mouse NOS III, Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). The autoradiographs were analyzed by scanning densitometry.

McCall et al. (1991) identified N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase.

Bauersachs et al. (1998, 1999) measured vascular reactivity in isolated rat aortic rings mounted in an organ bath (Föhr Medical Instruments, Seeheim Germany) for isometric force measurement and determined superoxide anion production by lucigenin-enhanced chemiluminescence and endothelial nitric ox-

ide synthase and soluble guanylyl cyclase expression by reverse transcription-polymerase chain reaction.

Von der Leyen et al. (1995) reported gene therapy inhibiting neointimal vascular lesions in rats. After denudation of the endothelium of carotid arteries by balloon injury, endothelial cell nitric oxidase expression in the vessel wall was restored by using the Sendai virus/liposome *in vivo* gene transfer technique.

Lund et al. (2000) found that gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits.

Mungrue et al. (2002) discussed lessons from murine genetic models on the role of NOS in heart failure.

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A.1.1.19**Inhibition of Rho Kinase****PURPOSE AND RATIONALE**

Rho is a member of the Ras-related family of small molecular weight GTP-binding proteins, and Rho works as a molecular switch by shuttling between the GDP-bound inactive form and the GTP-bound active form. Rho is involved in cell motility, cell adhesion, cytokinesis, Ras-induced transformation, transcriptional activation, and cell cycle progression. These actions, through Rho signaling, are mediated by downstream Rho effectors, such as the ROCK family of Rho-associated serine/threonine protein kinases. Studies with ROCK-specific inhibitors indicate that the ROCK pathway works in the contraction of vascular smooth muscle. Several studies indicate that Rho-kinase may be a novel therapeutic target in the treatment of cardiovascular disease (Kobayashi et al. 2002; Shimokawa 2002; Ito et al. 2003, 2004; Nakakuki et al. 2005; Budzyn et al. 2006; Winaver et al. 2006).

Ishizaki et al. (2000) described the pharmacological properties of a specific inhibitor of Rho-associated kinases.

PROCEDURE**Kinase assay**

Recombinant ROCK-I, ROCK-II, PKN, or citron kinase was expressed in HeLa cells as a Myc-tagged protein by transfection using lipofectamine, and was precipitated from the cell lysates by the use of 9E10 monoclonal anti-Myc antibody coupled to G protein-Sepharose (Ishizaki et al. 1997). Recovered immunocomplexes were incubated with various concentrations of [³²P]ATP and 10 μg of histone type 2 as substrates, in the absence or presence of various concentrations of test compounds at 30°C for 30 min in a total volume of 30 μl of the kinase buffer containing 50 mM HEPES-NaOH, pH 7.4, 10 mM MgCl₂, 5 mM MnCl₂, 0.02% Brij 35, and 2 mM dithiothreitol. PKCα was incubated with 5 μM [³²P]ATP and 200 μg/ml histone type 2 as substrates in the absence or presence of various concentrations of test compounds at 30°C for 10 min in a kinase buffer containing 50 mM Tris-HCl, pH 7.5, 0.5 mM CaCl₂, 5 mM magnesium acetate, 25 μg/ml phosphatidyl serine, 50 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate and 0.001% leupeptin in a total volume of 30 μl. Incubation was terminated by the addition of 10 μl of 4 × Laemmli sample buffer. After boiling for 5 min, the mixture was subjected to SDS-polyacrylamide gel electrophoresis on a 16% gel. The gel was stained with Coomassie

Brilliant Blue, and then dried. The bands corresponding to histone type 2 were excised, and the radioactivity was measured.

EVALUATION

K_i values were either determined by the double reciprocal plot or calculated from the equation $K_i = IC_{50}/(1 + S/K_m)$, where S and K_m are the concentration of ATP and the K_m value for ATP, respectively.

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A.1.1.20**Inhibition of Na⁺/H⁺ Exchange****PURPOSE AND RATIONALE**

Na⁺/H⁺ exchange was first described by Murer et al. (1976) in a study of intestinal and renal brush border vesicles. The plasma membrane Na⁺/H⁺ exchanger is an ubiquitous pH regulating cellular ion transport system. It is driven by the Na⁺ gradient and extrudes

protons from the cytosol in exchange for extracellular Na^+ ions (Aronson 1985; Frelin et al. 1988; Fliegel and Dyck 1995; Orlowski and Grinstein 1997; Wakabayashi et al. 1997; Dibrov and Fliegel 1998). Six mammalian Na^+/H^+ exchangers: NHE1, NHE2, NHE3, NHE4, NHE5 (Attaphitaya et al. 1999; Szabo et al. 2000), and NHE6 have been described (Tse et al. 1994; Orlowski 1999; Counillon and Pouyssegur 2000).

In cardiac tissue the exchanger has a major role in the control of intracellular pH. At the onset of cardiac ischemia and during reperfusion, Na^+/H^+ exchange is excessively activated by low intracellular pH. Since the deleterious Na^+ influx in this condition was found to originate mainly from Na^+/H^+ exchange (Frelin et al. 1984; Schömig et al. 1988), the exchanger seems to be responsible for an increase of cytosolic sodium in ischemic cells. The accumulation of intracellular Na^+ causes an activation of Na^+/K^+ ATPase (Frelin et al. 1984; Rasmussen et al. 1989) which in turn increases ATP consumption.

During ischemia the aerobic metabolism of glucose terminates in lactic acid. A vicious circle leads to a further decrease of intracellular pH and to a further activation of Na^+/H^+ exchange, resulting in energy depletion, cellular Na^+ overload and finally due to the coupling of Na^+ and Ca^{2+} transport via $\text{Na}^+/\text{Ca}^{2+}$ exchange, cellular Ca^{2+} overload (Lazdunski et al. 1985; Tani and Neely 1990; Scholz and Albus 1993). Especially in ischemic cardiac tissue, where Na^+/H^+ exchange is the predominant pH regulating ion transport system (Weissenberg et al. 1989), these pathological events can lead to increased excitability and precipitation of cellular death. Therefore, it is desirable to find potent and well tolerated inhibitors of Na^+/H^+ exchange which should be able to interrupt this vicious cycle, to conserve cellular energy stores and to diminish cellular excitability and necrosis during cardiac ischemia. Such effects have been found with relatively weak inhibitors of Na^+/H^+ exchange at high toxic doses, such as amiloride and ethyl isopropyl amiloride (Scholz et al. 1992).

The myocardial Na^+/H^+ exchanger is regarded as a therapeutic target for the prevention of myocardial ischemic and reperfusion injury and attenuation of postinfarction heart failure (Karmazyn et al. 2001).

More potent Na^+/H^+ exchange inhibitors showed beneficial effects on ischemia/reperfusion injury (see A.5.0.7 and A.5.0.8) in rats (Aye et al. 1997; Myers et al. 1998; Aihara et al. 2000), dogs (Gumina et al. 1998, 2000) and pigs (Portman et al. 2001). Heart hypertrophy and heart failure after myocardial infarction

is reduced (Yoshida and Karmazyn 2000; Kusumoto et al. 2001). Ischemia-induced apoptosis in isolated rat hearts is attenuated by sodium-hydrogen exchange inhibitors (Chakrabarti et al. 1997).

Linz and Busch (2003) demonstrated the effects of NHE-1 inhibition from protection during acute ischemia/reperfusion to prevention of myocardial remodeling.

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A.1.1.20.1

Inhibition of Na⁺/H⁺ Exchange in Thrombocytes

PURPOSE AND RATIONALE

The inhibition of Na⁺/H⁺ exchange has been studied in platelets by measuring the optical density after osmotic cell swelling (Rosskopf et al. 1991).

PROCEDURE

About 5 ml blood is withdrawn by venipuncture from human donors or from the vena jugularis externa of

Beagle dogs or from the aorta of anesthetized Wistar rats (weighing 250–350 g). Coagulation is inhibited by 0.8 ml citrate acid dextrose (65 mM citric acid, 11 mM glucose, 85 mM trisodium citrate). Platelet-rich plasma (PRP) is obtained by centrifugation of whole blood at 90 g for 10 min at room temperature. Platelet count is measured, e. g., with a Casey 1 multichannelyser (Schärfe System, Reutlingen, Germany).

Each of the experiments is performed with 10–50 µl PRP containing 20 × 10⁶ platelets in a volume of 100 µl with saline. To activate Na⁺/H⁺ exchange in the platelets by intracellular acidification, 500 µl propionate buffer (135 mM Na-propionate, 1 mM HCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 20 mM HEPES, pH 6.7, 22°C) are added to the PRP/NaCl solution. Swelling of the platelets results in a decrease of optical density which can be measured with an aggregometer, e. g. with a Turbitimer (Behringwerke, Marburg, Germany). The system is activated photo-metrically by the addition of the propionate buffer to the cuvette. The experiments are performed with and without the addition of the Na⁺/H⁺ exchange inhibitor to be tested. The inhibitors are added in concentrations between 10⁻⁴ and 10⁻⁸ mol/l. 5-(N-ethyl-N-isopropyl)amiloride (EIPA) is used as standard. During the experiments all solutions are kept at 22°C in a temperature controlled water bath.

EVALUATION

Results are given as means ± SD. Student's *t*-test is employed for statistical evaluation. IC₅₀ values are calculated from dose-response curves.

REFERENCES AND FURTHER READING

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A.1.1.20.2

Inhibition of Na⁺/H⁺ Exchange in Cholesterol Activated Rabbit Erythrocytes

PURPOSE AND RATIONALE

The inhibition of Na⁺/H⁺ exchange has been studied in cholesterol activated rabbit erythrocytes by flame photometry of sodium (Scholz et al. 1992, 1993).

PROCEDURE

White rabbits (New Zealand strain, Ivanovas) are fed with a rabbit standard chow with 2% cholesterol for

6 weeks to increase the Na^+/H^+ exchange (Scholz et al. 1990) and to make the erythrocytes suitable for measurement of sodium influx via Na^+/H^+ exchange by flame photometry. Blood is drawn from the ear artery of the rabbits and coagulation prevented with 25 IU/ml potassium heparin. The hematocrit of the samples is determined in duplicate by centrifugation. Aliquots of 100 μl are taken to measure the initial sodium content of the erythrocytes.

To determine the amiloride sensitive sodium influx into erythrocytes, 100 μl of each blood sample are added to 5 ml of buffer made hyperosmolar by sucrose (140 mM NaCl, 3 mM KCl, 150 mM sucrose, 0.1 mM ouabain, 20 mM tris-hydroxymethylaminomethane, pH 7.4) and incubated for 60 min at 37°C. Subsequently, the erythrocytes are washed three times in ice-cold MgCl_2 -ouabain-solution (112 mM MgCl_2 , 0.1 mM ouabain).

For determination of intracellular sodium content, the cells are hemolyzed in distilled water, the cell membranes are centrifuged and the sodium concentration of the haemolysate is measured by flame photometry. Net influx of sodium into the erythrocytes is calculated from the difference between the initial sodium content and the sodium content after incubation. Amiloride-sensitive sodium influx is calculated from the difference between sodium content of erythrocytes incubated with and without amiloride (3×10^{-4} M). Each experiment is done with the erythrocytes from 6 different animals. In each case, the comparison of Na^+ contents is based on erythrocytes from the same animal. Doses between 10^{-4} and 10^{-7} M of the inhibitor are tested.

EVALUATION

Statistical analysis of the data obtained is performed with Student's *t*-test for paired groups. IC_{50} values are calculated from dose-response curves.

REFERENCES AND FURTHER READING

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A.1.1.20.3

Sodium Influx into Cultured Cardiac Myocytes

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in cultured cardiac myocytes (Scholz et al. 1992).

PROCEDURE

Rat myocardial cells are isolated from hearts of neonatal rats by trypsin digestion. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's Minimum Essential Medium (DMEM, GIBCO) in an atmosphere containing 10% CO_2 . After confluence, the cells are used for measurement of $^{22}\text{Na}^+$ influx. The cells are washed twice with Krebs-Ringer-solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (Choline chloride 130 mM, CaCl_2 1.5 mM, KCl 5 mM, MgCl_2 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37°C in the same buffer with added 0.1% bovine serum albumin (BSA) and 10 mM/l glucose. The culture dishes are then incubated for another 10 min with Na^+ -propionate for cytosolic acidification and stimulation of Na^+/H^+ exchange. The compounds are dissolved in 500 μl /dish KRB in which 50% of the sodium chloride has been replaced by choline chloride containing additionally 2 $\mu\text{Ci}/\text{ml}$ $^{22}\text{Na}^+$ -bicarbonate, and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). After the stimulation period, sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM MgCl_2 , 10 mM Tris, pH 7.0). Subsequently, the cells are lysed with 250 μl trichloroacetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 3×10^{-4} and 10^{-8} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

EVALUATION

Mean values \pm SD are compared with Student's *t*-test. IC_{50} values are calculated from dose-response curves.

REFERENCES AND FURTHER READING

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A.1.1.20.4**Inhibition of Na⁺/H⁺ Exchange into Cultured Aortic Endothelial Cells****PURPOSE AND RATIONALE**

The inhibition of Na⁺/H⁺ exchange has been studied in endothelial cells (Scholz et al. 1993) by measuring the ²²Na⁺ influx.

PROCEDURE

Bovine aortic endothelial cells (BAEC) are isolated by dispase digestion from bovine aorta obtained from animals killed at the local slaughter house. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's Minimum Essential Medium (DMEM, GIBCO) in an atmosphere with 10% CO₂. Three days after confluence the cells are used for measurement of ²²Na⁺ influx. The cells are washed twice with Krebs-Ringer solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (Choline chloride 130 mM, CaCl₂ 1.5 mM, KCl 5 mM, MgCl₂ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37°C in the same buffer with added 0.1% bovine serum albumin (BSA) and 10 mM glucose. To stimulate Na⁺/H⁺ exchange the culture dishes are incubated for another 10 min with 500 μl/dish KBR in which all sodium chloride has been replaced by 65 mM each of choline chloride and Na⁺-propionate or with KBR in which 50% of the sodium chloride has been replaced by choline chloride for unstimulated controls. In addition, the buffer contains 2 μCi/ml ²²Na⁺ and the test compounds or the standard. After the stimulation period, the sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM MgCl₂, 10 mM Tris, pH 7.0). Subsequently, the cells are lysed with 250 μl trichloroacetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 10⁻⁵ and 10⁻⁷ mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

EVALUATION

Mean values ± SD are compared with Student's *t*-test. IC₅₀ values are calculated from dose-response curves.

MODIFICATIONS OF THE METHOD

Ewart et al. (1997) studied lipoprotein lipase activity in cultured rat cardiomyocytes in the presence of insulin and dexamethasone.

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A.1.1.20.5**NHE Activity Measured by Intracellular pH in Isolated Ventricular Myocytes****PURPOSE AND RATIONALE**

Changes of the intracellular pH of cultured bovine endothelial cells have been fluorometrically monitored using the pH-dye 2',7'-bis(carboxyethyl)carboxyfluorescein (BCECF) by Kitazano et al. (1988). This method has been used to study the activity of inhibitors of Na⁺/H⁺ exchange (Scholz et al. 1995).

PROCEDURE

For preparation of isolated rat ventricular muscular cells (Yazawa et al. 1990), hearts of male Wistar rats are dissected, mounted on a Langendorff apparatus and perfused first at 37°C for 3 min with Tyrode solution adjusted to pH 7.4, second for 5–7 min with nominally calcium free Tyrode solution and finally with calcium free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma type I). After 15–20 min collagenase treatment, the heart is washed with storage solution (composition in mmol/L: KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH₂PO₄ 20, MgCl₂ 3, glucose 20, HEPES 10, and EGTA 0.5, pH 7.4). The ventricles are cut into small pieces and myocytes are dispersed by gently shaking and finally by filtration through a nylon mesh (365 μm). Thereafter, the cells are washed twice by centrifugation at 600–1000 rpm for 5 min and kept at 4°C until use. For the pH recovery experiment the cells are loaded with the membrane permeable acetoxymethyl ester (AM) form of the fluorescent indicator 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). BCECF-AM is dissolved in DMSO and diluted to a 1.25 μM storage solution. Cardiomyocytes are loaded in this solution for 30 min at room temperature and are then centrifuged and resuspended in storage solution. The measure-

ments are performed in bicarbonate-free NaCl solution (NaCl 140, KCl 4.7, CaCl₂ 1.3, MgCl₂ 1, glucose 10, and HEPES 10 mM/L, pH 7.4) at 34°C using an apparatus according to Nitschke et al. (1991). The pH-dependent signal of BCECF is obtained by illuminating at 490 and 437 nm and dividing the emitted light signals (520–560 nm). The background signal, determined by closing the shutter, is subtracted from the total signal. The autofluorescence determined by illuminating unloaded cells can be ignored. In order to investigate the function of the Na⁺/H⁺ exchange system, the intracellular pH (pH_i) of the cells is decreased by the NH₄Cl prepulse technique and the rate of return to resting pH_i is determined. Test compounds are dissolved in the incubation medium. For each test concentration, the recovery of pH_i is first recorded in control NaCl solution.

EVALUATION

Data are analyzed by fitting a straight line to the initial (5 min) data points of the pH recovery curve. For statistical presentation, the slopes of the linear curves are demonstrated. All reported data are presented as means ± SEM. Statistical comparisons are made using either a paired or unpaired *t*-test.

MODIFICATIONS OF THE METHOD

The pH-sensitive fluorescence dye C-SNARF-1 (= carboxy-semi-naphtho-rhoda-fluor 1) was used by Yasutake et al. (1996), Shipolini et al. (1997), and Yokoyama et al. (1998).

Fischer et al. (1999) tested new drugs for the Na⁺/H⁺ exchanger in Chinese hamster ovary cells which are enriched with the NHE-1 isoform of the Na⁺/H⁺ anti-porter. The Na⁺/H⁺ exchanger was stimulated with NaCl and the rate of extracellular acidification was quantified with the Cytosensor.

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NHE Subtype Specificity

PURPOSE AND RATIONALE

Molecular identification of mammalian Na⁺/H⁺ exchanger subtypes has been pioneered by Pouysségour and coworkers (Sardet et al. 1989) who used genetic complementation of fibroblast cell lines that lack all endogenous NHEs. Schwark et al. (1998) studied an inhibitor of Na⁺/H⁺ exchanger subtype 3 in various cell types.

PROCEDURE

cDNAs for the NHE subtypes human NHE1, rabbit NHE2, rat NHE3 (Pouysségour) or cloned by reverse transcriptase-polymerase chain reaction from human kidney mRNA are used. These cDNAs are cloned into the mammalian expression vector pMAMneo and transferred into the NHE-deficient mouse fibroblast cell line LAP1. Cells expressing the NHE subtypes are selected by the acid load survival method (Sardet et al. 1989). Clonal cell lines for each subtype are used for intracellular pH (pH_i) recovery after acid load. For studies of pH_i recovery (Faber et al. 1996), cells are scraped off the culture dishes washed and incubated with 5 μmol/l BCECF-AM [2',7'-bis(2-carboxyethyl)-5,6-carboxy-fluorescein-acetoxy-methyl ester] for 20 min at 37°C in a buffer containing 20 mM NH₄Cl. The cells are then washed to remove extracellular dye and resuspended in the loading buffer without BCECF-AM. Intracellular acidification is induced by addition of 975 μl NH₄Cl-free and HCO₃⁻-free solution (so-called recovery medium: HCO₃⁻-free to inhibit the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger of LAP1 cells) to an aliquot of cells (≈25,000 cells). The pH_i recovery is recorded with a dual-grating Deltascan single-photon counting fluorometer (Photon Technology International, South Brunswick, NJ, USA) with exci-

tation wavelength of 505 nm and 440 nm and an emission wavelength of 535 nm. The measurement time varies between subtypes (120 s for NHE1, 300 s for NHE2, 180 s for NHE3). The inhibitors are first dissolved in DMSO, diluted in recovery medium and added in a volume of 975 μ l to this medium.

A cloned opossum kidney cell line (Helmle-Kolb et al. 1990) is used additionally. Cells are grown as a monolayer in growth medium (1:1 mixture of nutrient mixture Ham F12 and Dulbecco's modified medium Eagle with 10% fetal calf serum). For subcultivation and pH-recovery experiments, the cells are detached from the surface of the culture vessels with trypsin-EDTA solution (2.5 g trypsin+0.2 g EDTA per liter in Dulbecco's phosphate-buffered saline) and suspended in growth medium. Measurement time in pH_i recovery experiments is 400 s.

Porcine renal brush-border membrane vesicles (BBMV) prepared by a Mg²⁺ precipitation technique are loaded with 150 mmol/l NaCl, 5 mmol/l HEPES/Tris, pH 7.0, and pre-incubated for 10 min at 37°C with various concentrations of NHE inhibitors. Intravesicular acidification through Na⁺/H⁺ exchange is started by diluting BBMV into Na⁺-free buffer (150 mmol/l tetramethylammonium chloride, 5 mmol HEPES/Tris, pH 7.0) containing the appropriate concentrations of the NHE inhibitors and the fluorescent Δ pH indicator acridine orange (12 μ mol/l). The fluorescence changes of acridine orange are recorded continuously by a Hitachi F-2000 spectrofluorometer at 495 nm excitation and 525 nm emission wavelength. The initial acridine orange fluorescence quenching in controls (no inhibitor) is set to 100%.

EVALUATION

Values are presented as means \pm SD (four measurement per concentration). The *IC*₅₀ values and Hill coefficients are calculated using the Sigma plot software. Statistical significance is calculated by means of the distribution-independent *H*-test and non-parametric *U*-test. *P* < 0.05 is considered as significant.

MODIFICATIONS OF THE METHOD

Counillon et al. (1993), Scholz et al. (1995) determined the NHE subtype specificity of Na⁺/H⁺ antiporters by their ability to inhibit initial rates of amiloride sensitive ²²Na⁺ uptake in fibroblast cell lines separately expressing the NHE-1, NHE-2 and NHE-3 isoforms.

Ko et al. (2004) determined the inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure-activity relationships.

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Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and/or cGMP. They function with adenylyl and guanylyl cyclases to regulate the amplitude and duration of responses triggered by the second messengers cAMP and cGMP. The enzyme phosphodiesterase (PDE) exists in various forms. At least 11 families of phosphodiesterases have been identified (Torphy and Page 2000; Francis et al. 2001; Maurice et al. 2003; Lugnier 2006). The properties and functions of GAF domains in cyclic nucleotide phosphodiesterases are reviewed by Zoraghi et al. (2004).

Hofmann et al. (2006) reviewed the nomenclature and structure-function relationships of cyclic nucleotide-regulated channels.

Mongillo et al. (2004) reported fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes, revealing distinct functions of compartmentalized phosphodiesterases. Studying real-time monitoring of PDE2 activity in live cells, Nikolaev et al. (2005) found that hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis.

Snyder et al. (2005) studied the role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis.

Zhang et al. (2004) described a glutamine switch mechanism for nucleotide selectivity by phosphodiesterases.

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Inhibition of Phosphodiesterase

PURPOSE AND RATIONALE

The inhibition of cAMP-PDE and cGMP-PDE by various test compounds can be measured using a two-step radioisotopic procedure.

PROCEDURE

Materials

[8-³H]cAMP (28 Ci/mmol), [8-³H]-cGMP (15 Ci/mmol) and [U¹⁴C]guanosine (528 mCi/mmol) are obtained from Du Pont de Nemours (Paris, France). Unlabelled cyclic nucleotides, 5'-nucleotidase (Ophiophagus hannah venom) are from the Sigma Chemical Co. (La Verpillère, France).

Tissue preparations

Male Sprague-Dawley rats (250–300 g) are decapitated. Hearts are perfused with 0.15 M NaCl through the aorta to remove the blood. The ventricles are minced in 5 vol. of 10 mM Tris-HCl buffer containing 0.32 M sucrose, 1 mM EDTA, 5 nM DTT and 0.1 mM PMSF at pH 7.5. The suspension is homogenized in a glass-glass Potter-Elvehjem. The homogenate is then centrifuged at 105,000 g for 60 min. The 105,000 g supernatant is stored at –75°C until injection on the HPLC column.

Isolation of PDEs

The cytosolic fraction from rat ventricles (5–8 mg of protein) is loaded at the rate of 1 ml/min on a Mono Q HPLC column which has been previously equilibrated with buffer A (50 mM Tris-HCl, 2 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5). Under these conditions, greater than 95% of the PDE activity is bound to the column. PDE activity is eluted at a flow rate of 1 ml/min using the following step by step and linear gradients of NaCl in buffer A: 25 ml of 0.16 M NaCl, 20 ml of 0.23 M NaCl, 30 ml from 0.23 to 0.29 M NaCl, 15 ml of 0.29 M NaCl, 30 ml from 0.29 to 0.50 M NaCl. The separation is done at 4°C. Fractions of 1 ml are collected and stored at –75°C in the presence of 20% glycerol. The fractions are tested for PDE activity, and the peaks containing the different isoenzymes are identified. Fractions containing preferentially one isoenzyme are pooled.

PDE Assay

PDE activity is assayed by a two-step radioisotopic procedure according to Thompson et al. (1974), Boudreau and Drummond (1975), Prigent et al. (1981). cAMP-PDE and cGMP-PDE activities are measured with a substrate concentration of 0.25 μM. To evaluate the cGMP-stimulated PDE activity, assays are performed with 5 μM cAMP in the absence or presence of 5 μM cGMP. Xanthine derivatives are dissolved in DMSO. The stock solutions are appropriately diluted with 40 mM Tris-HCl buffer so that the final DMSO concentration in the PDE assay does not exceed 1%. At this concentration, DMSO has no significant effect on the PDE activity of any of the fractions. The inhibitory potency of the xanthine derivatives is examined on each separated isoform.

EVALUATION

The *IC*₅₀ values (concentration of a drug which inhibit 50% of the enzymatic activity) are calculated by plotting the percentage of residual enzymatic activity ver-

sus the logarithmic concentration of the drug. Confidence limits (95%) for the IC_{50} values are determined by linear regression analysis.

MODIFICATIONS OF THE METHOD

Phosphodiesterase activity can be determined using ^{32}P -labelled GTP (Reinsberg 1999). The reaction is performed using an Eppendorf cup with 10 mM GTP, the test solution and [^{32}P]GTP. The reaction is started with 1 mM sodium nitroprusside solution. After an incubation period of 20 min at 37°C, the reaction is stopped with 500 μ l of 120 mM sodium carbonate and 400 μ l of zinc acetate, whereby the zinc carbonate and the substrate GTP are almost (about 90%) all precipitated, and so separated from cGMP. The precipitate is separated by centrifugation and the supernatant submitted to chromatography on an aluminum oxide column. The column is first treated with Tris HCl buffer. From the supernatant, 900 ml is applied. After two washing periods, the cGMP is eluted in five fractions. These fractions are measured and the highest activities are pooled.

The PDE activity is calculated from the difference between applied [^{32}P]GTP minus unaltered [^{32}P]GTP.

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Description of Phosphodiesterase Isoforms

Phosphodiesterase 1

PDE1 family variants are activated upon Ca^{2+} /calmodulin binding (Kakkar et al. 1999; Goraya and Cooper 2005; Sharma et al. 2006). The PDE1 subfamily consists of three different gene products (PDE1A, PDE1B, and PDE1C) which differ in their regulatory properties, substrate affinities, specific activities for calmodulin, tissue distribution, and molecular weights (Yan et al. 1996; Yu et al. 1997). PDE1 is present in brain, cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells (Maurice et al. 2003). Inhibition has been described for nimodipine (Epstein et al. 1982) and for vinpocetine (Hagiwara et al. 1984).

Phosphodiesterase 2

A single *PDE2* gene encodes three PDE2 variants (Rosman et al. 1997). Martinez et al. (2002) studied the crystal structure of murine PD2A and found that the two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and cGMP binding. PDE2 is found in various areas of the brain (Lugnier 2006), in adrenal medulla, heart, rat ventricle (Yanaka et al. 2003), liver, and brown adipose tissue (Coudray et al. 1999). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was shown to specifically act on PDE2 by inhibiting cCMP-activated PDE2 (Podzuweit et al. 1995).

Chambers et al. (2006) described a new chemical tool for exploring the physiological function of the PDE2 isozyme.

Phosphodiesterase 3

The PDE3 family is composed of two genes, *PDE3A* and *PDE3B*. *PDE3A* mRNA is enriched in blood vessels, heart, megakaryocytes and oocytes, whereas *PDE3B* is highest in adipocytes, hepatocytes, brain, renal collecting duct epithelium, and developing spermatocytes (Reinhardt et al. 1995). PDE3 inhibitors have been extensively investigated and developed as non-glycoside, non-sympathomimetic, cardiotoxic agents for the treatment of heart failure. Milrinone is the most studied and most extensively used PDE3 inhibitor and is used in the acute treatment of heart failure (Cruickshank 1993). Trequinsin (HL 725) inhibits PDE3 in a nanomolar range (Ruppert and Weithmann 1982); it also inhibits PDE1, PDE2, and PDE4 in submicromolar concentrations (Stoclet et al. 1995).

Boswell-Smith et al. (2006) studied the pharmacology of two long-acting trequinsin-like phosphodiesterase 3/4 inhibitors, RPL554 and RPL565.

Hambleton et al. (2005) studied isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium.

Masciarelli et al. (2004) described mice deficient of cyclic nucleotide phosphodiesterase 3A as a model of female infertility.

Adachi et al. (2005) reported the effects of a phosphodiesterase 3 inhibitor, olprinone, on rhythmical change in the tension of human gastroepiploic artery.

Abbott and Thompson (2006) performed analysis of anti-PDE3 activity of 2-morpholinochromone derivatives revealing multiple mechanisms of anti-platelet activity.

Phosphodiesterase 4

PDE4 is mainly present in the brain (Houslay et al. 1998), inflammatory cells (Tenor and Schudt 1996), cardiovascular tissues (Stoclet et al. 1995), and smooth muscles, but is lacking in the platelets. Four *PDE4* genes (*PDE4A–D*) yield a large number of distinct PDE4 variants. These enzymes, which result from the use of alternate promoters and extensive splicing of PDE4 mRNAs, are stratified into long or short forms (Conti et al. 2003).

One *PDE4A*, three *PDE4B* (PDE4B1, PDE4B2, and PDE4B3), and three *PDE4D* (PDE4D1, PDE4D2, PDE4D3) variants are expressed in rat and human cardiac tissue (Houslay and Adams 2003). Two *PDE4D* gene-derived variants, PDE4D3 and PDE4D6, are expressed in human and rat aortic, mesenteric, and femoral contractile/quiescent and synthetic/activated vascular smooth muscle cells (Liu et al. 2000).

The role of cAMP-specific PDE4 phosphodiesterases in cellular signaling was reviewed by Houslay and Adams (2003) and Conti et al. (2003).

Huai et al. (2003) studied the three-dimensional structures of PDE4D in a complex with roliprams and the implication for inhibitor selectivity.

Baillie et al. (2003) found that β -arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β -adrenoceptor switching from G_s to G_i .

The antidepressant compound rolipram (ZK 62711) is a selective inhibitor of PDE4 (Schwabe et al. 1976; Komasa et al. 1989). O'Donnell and Zhang (2004) described the antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). Many analogs were studied as PDE4 inhibitors for treatment of asthma and chronic obstructive pulmonary disease (COPD) as well

as anti-inflammatory drugs; however, most failed due to emetic side-effects (Giembycz 2002).

Phosphodiesterase 5

Selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (**PDE5**) were found to be effective in the treatment of erectile dysfunction in men (Jeremy et al. 1997; Ballard et al. 1998; Chuang et al. 1998; Stief et al. 1998; Corbin and Francis 1999; Turko et al. 1999; Wallis et al. 1999; Hosogai et al. 2001; Rotella 2001; Saenz de Tejada et al. 2001; Ukita et al. 2001).

The PDE5 family consists of a single *PDE5* gene that can encode three distinct proteins (PDE5A1–3) (Loughney et al. 1998).

Wang et al. (2001) characterized type 5 phosphodiesterases in the corpus cavernosum of several species.

Qiu et al. (2000) demonstrated that rabbit corpus cavernosum smooth muscle shows a different phosphodiesterase profile than human corpus cavernosum.

Kim et al. (2001) compared the inhibition of cyclic GMP hydrolysis in human corpus cavernosum smooth muscle cells by vardenafil with that by sildenafil.

The discovery of novel, potent, and selective PDE5 inhibitors was reported by Bi et al. (2001).

Corbin et al. (2004) described the structural basis for the higher potency of vardenafil compared with sildenafil in inhibiting cGMP-specific phosphodiesterase-5 (PDE5).

PDE5 inhibitors such as sildenafil can be used in the treatment of pulmonary arterial hypertension (Galié et al. 2005).

Cohen et al. (1996) found that inhibition of cGMP-specific phosphodiesterase selectively vasodilates the pulmonary circulation in rats made chronically hypoxic by exposure to a simulated high altitude. Chronic hypoxia augments protein kinase G-mediated Ca^{2+} desensitization in pulmonary vascular smooth muscle through inhibition of RhoA/Rho kinase signaling (Jernigan et al. 2004).

Thompson et al. (2001) studied the effect of sildenafil on corpus cavernosal smooth muscle relaxation and cGMP formation in the diabetic rabbit.

Tantini et al. (2005) found an antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells.

Other Phosphodiesterases

The families of **PDE6 to PDE11** have been identified as a result of bioinformatics-based genomic screening (Soderling and Beavo 2000). The search for inhibitors is just beginning.

Gillespie and Beavo (1989) discussed inhibition and stimulation of photoreceptor phosphodiesterases (later on termed **PDE6**) by dipyrindamole and M&B 22,948.

D'Armours et al. (1999) investigated the potency and mechanism of action of E4021, a type 5 phosphodiesterase isozyme-selective inhibitor, on the photoreceptor phosphodiesterase.

Zhang et al. (2005) described the efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (**PDE6**) in retinal photoreceptors.

Smith et al. (2004) reported the discovery of a selective inhibitor of **phosphodiesterase 7**.

The discovery of thiadiazoles as a novel structural class of potent and selective **PDE7 inhibitors** was reported by Vergne et al. (2004a, 2004b).

Lorthiois et al. (2004) and Bernadelli et al. (2004) described spiroquinazolinones as potent and selective **PDE7 inhibitors**.

The first potent and selective **PDE9 inhibitor** was characterized by Wunder et al. (2005) using a cGMP reporter cell line.

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A.1.1.22

Stimulation of Heart Membrane Adenylate Cyclase

PURPOSE AND RATIONALE

Metzger and Lindner (1981) discovered that the positive inotropic and vasodilatory effects of forskolin were correlated with the stimulation of adenylate cyclase and cAMP-dependent protein kinase. Subsequent studies by Seamon (1981) demonstrated that forskolin, unlike hormones, guanine nucleotides, fluoride or cholera toxin could stimulate cyclase activity in the absence of the guanine nucleotide regulatory protein. Since those reports, hundreds of papers have been published on the effects of forskolin in numerous mammalian organ and cell systems. Several comprehensive review articles have also been published (Seamon and Daly 1981, 1983; Daly 1984).

While forskolin has proven to be an invaluable research tool for investigations of adenylate cyclase systems (Salomon et al. 1974; Seamon et al. 1981, 1983), reports on its effects on cardiovascular (Lindner et al. 1978), pulmonary (Chang et al. 1984) and ocular physiology (Caprioli and Sears 1983; Caprioli 1985) suggest a therapeutic potential (Seamon 1984) as well.

Described here is an *in vitro* assay which can be used to compare the potency of forskolin, forskolin analogs or other direct or indirect adenylate cyclase activators for the stimulation of adenylate cyclase in heart membranes. The purpose of this assay is to determine and compare the potency of direct or indirect activators of adenylate cyclase for an ability to stimulate heart membrane adenylate cyclase *in vitro*.

PROCEDURE

The hearts of the Wistar rat, Hartley guinea pig, golden Syrian hamster or cardiomyopathic hamster (CHF-146) are used as a source of adenylate cyclase for this assay.

Reagents

1. 0.5 M Tris buffer, pH 7.4
2. 0.05 M Tris buffer, pH 7.4, containing 0.1 M CaCl₂
3. Tris buffer mixture
0.05 M Tris buffer, containing 1 mM IMBX (isobutylmethylxanthine), 0.2 mM EGTA, 5 mM MgCl₂, 0.5 mM ATP (Na₂ATP × 3 H₂O) and 20 mM creatine phosphate (Na₂ Creatine-PO₄ × 5 H₂O) (final concentrations in the incubation media).
4. Creatine phosphokinase (ATP:Creatine N-Phosphotransferase, EC 2.73.2), type I, from rabbit muscle is obtained from Sigma Chemical Co.
The concentration of enzyme in the incubation media is 40 U/ml.
5. Test compounds

For most assays, a 30 mM stock solution is made up in a suitable solvent (ethanol, ethyl acetate or DMSO for most forskolin analogs). Serial dilutions are made such that the final concentration in the assay ranges from 3×10^{-4} to 3×10^{-7} M. The concentration of vehicle in the assay is 1%.

Tissue Preparation

One entire heart from rat, guinea pig or hamster is dissected, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 7.4 containing 0.1 mM CaCl₂ (reagent 2) using a Brinkman Polytron (setting 7 for 15 s). The homogenate is centrifuged at 10,000 g for 20 min at 4°C. The resulting pellet is resuspended in 50 volumes of homogenizing buffer and recentrifuged as before. The supernatant of this spin is discarded and the resulting pellet is finally resuspended in 10 volumes of the homogenizing buffer, for rat and hamster and 60 volumes for guinea pig tissue. This final preparation is filtered through a thin layer of gauze and kept on ice until used in the assay. The protein concentration is approximately 250–350 mg/ml.

Protein concentrations from an aliquot of the tissue suspension are determined on the day of the experiment by the method of Bradford (1976) using the Bio-Rad assay kit.

Assay

- | | |
|--------|---------------------------------------------------|
| 300 µl | Tris buffer mixture, pH 7.4 (reagent 3) |
| 50 µl | creatine phosphokinase |
| 5 µl | vehicle or appropriate concentration of test drug |
| 95 µl | H ₂ O |
| 50 µl | tissue suspension |

The tubes are incubated for 5 min at 37°C and the reaction is then stopped by placing the tubes into a boiling

water bath for 4 min. The tubes are then centrifuged at 1000 g for 10 min and cAMP levels determined in a 15 µl aliquot of the supernatant using a radioimmunoassay kit (Code TRK432) obtained from Amersham according to the manufacturer's protocol.

Test principle: The method is based on the competition between unlabeled cAMP and a fixed quantity of ³H-labelled cAMP for binding to a protein which has high specificity and affinity for cyclic AMP. The amount of labelled cAMP-protein complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. The concentration of cAMP in the unknown is determined by comparison with a linear standard curve.

EVALUATION

The data are expressed as pmol cAMP/mg protein/min and dose-response stimulation curves are subjected to logic analysis to determine the concentration of compound which exhibits 50% of maximal stimulation (ED₅₀).

The β-blocking activities of compounds can be determined by their activities, by which they counteract the isoproterenol and GTP induced stimulation of rat heart membrane bound adenylate cyclase (Greenslade et al. 1979).

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A.1.1.23

³H-Forskolin Binding Assay

PURPOSE AND RATIONALE

This assay is used to identify compounds which demonstrate high affinity [nM] for association with forskolin binding sites *in vitro* as a preliminary screen in conjunction with stimulation of adenylate cyclase to determine the potential for cardiac chronotropic and inotropic and other effects of forskolin. Guinea pig heart tissue and rat brain tissue are used as sources of binding assays.

PROCEDURE FOR GUINEA PIG HEART TISSUE

A. Reagents

- 0.05 M Tris-HCl buffer, pH 7.4
- 0.05 M Tris-HCl, pH 8.0 containing 1 mM EGTA, 1 mM MgCl₂ and 0.32 M sucrose
- [³H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear. Final concentration in the assay is approximately 15 nM.
- Forskolin and test compounds
Forskolin and forskolin analogs are diluted to 40 μM. A 100 μl addition of this solution to the final incubate of 200 μl tissue and 100 μl ³H-forskolin results in a final concentration of 10⁻⁵ M.

Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10⁻⁵ to 10⁻¹¹ M with each concentration being done in triplicate.

B. Tissue preparation

Male Hartley guinea pigs (300–350 g) are sacrificed by decapitation. The heart is immediately removed, weighed, rinsed, diced and homogenized in 10 volumes of ice-cold 0.05 M buffer (reagent 2) using a Polytron homogenizer (setting 10, 30 s). The resulting homogenate is centrifuged at 12,000 g for 15 min at 4°C. The clear supernatant of this spin is discarded, the remaining pellet (P₂) is resuspended in 5 volumes of the same ice-cold buffer and rehomogenized. This final suspension (approximately 0.8–1.0 mg protein/ml) is kept on ice until use. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

To generate a dose-response inhibition curve, ³H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

200 μl tissue suspension

100 μl ³H-forskolin

100 μl appropriate concentration of forskolin or forskolin analog, or buffer

Tubes are incubated at 30°C for 10 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 (reagent 1) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed 3 times with 5 ml aliquots of Tris-HCl buffer (reagent 1), added to 10 ml scintillation cocktail and analyzed for radioactivity.

PROCEDURE FOR RAT BRAIN TISSUE

A. Reagents

- 0.32 M sucrose buffer
- 0.05 M Tris-HCl buffer, pH 7.5
- [³H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear. Final concentration in the assay is approximately 10 nM.
- Forskolin and test compounds
Forskolin and forskolin analogs are diluted to 40 μM. A 100 μl addition of this solution to the final incubate of 200 μl tissue and 100 μl ³H-forskolin results in a final concentration of 10⁻⁵ M.

Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation

Male Sprague-Dawley rats (200–250 g) are sacrificed by decapitation. The brain is rapidly removed and dissected on ice. Striata are homogenized in 50 volumes of ice-cold 0.32 M sucrose buffer (reagent 1) using a Polytron homogenizer (setting 7, 15 s). The resulting homogenate is centrifuged at 1000 *g* for 10 min at 0–4°C. The supernatant is retained and recentrifuged at 20,000 *g* for 10 min at 0–4°C. The clear supernatant of this spin is discarded and the remaining pellet (P₂) is resuspended in ice-cold Tris-HCl buffer, pH 7.5 such that the final protein concentration is approximately 3.0–4.0 mg/ml. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

To generate a dose-response inhibition curve, ³H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

200 μl tissue suspension

100 μl ³H-forskolin

100 μl appropriate concentration of forskolin or forskolin analog or buffer

Tubes are incubated at 23°C for 60 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 (reagent 2) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed three times with 5 ml aliquots of Tris-HCl buffer (reagent 2), added to 10 ml scintillation cocktail and analyzed for radioactivity.

EVALUATION

Specific binding is defined as the difference between binding of ³H-forskolin in the absence and presence of 10 μM forskolin and represents 85–90% of total binding at 10 nM ³H-forskolin.

Results of the dose-response inhibition curves are analyzed by determining the concentration of competing compound which inhibits 50% of the ³H-forskolin binding sites (*IC*₅₀). This value is determined by computer-derived log-probit analysis.

The activity of various forskolin analogs are based on *IC*₅₀ values and are categorized as follows:

| | <i>IC</i> ₅₀ [M] |
|-----------------------|------------------------------------|
| 0 = Not determined | |
| 1 = No activity | >10 ⁻⁵ |
| 2 = Slight activity | 10 ⁻⁵ –10 ⁻⁶ |
| 3 = Moderate activity | 10 ⁻⁶ –10 ⁻⁷ |
| 4 = Marked activity | <10 ⁻⁷ |

To compare the activity of various compounds from experiment to experiment, an inhibition constant (*K*_I) is determined as described by Cheng and Prusoff (1973). The *K*_I is determined from the equation:

$$K_I = IC_{50}/1 + LC/K_D$$

*IC*₅₀ = concentration of competing compound which inhibits 50% of the ³H-forskolin binding sites

LC = determined ³H-forskolin concentration (approximately 10 nM)

*K*_D = dissociation of affinity constant for ³H-forskolin determined previously to be approximately 13.4 nM for rat striatum and 196 nM for guinea pig heart

Standard data:

Binding inhibition values for forskolin

| | Striatum | Heart |
|-------------------------|---------------|---------------|
| <i>IC</i> ₅₀ | 43.1 ± 4.9 nM | 34.2 ± 5.0 nM |
| <i>K</i> _I | 26.0 ± 3.1 nM | 31.6 ± 4.6 nM |

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A.1.1.24 Patch Clamp Technique²

PURPOSE AND RATIONALE

The introduction of the patch clamp technique (Neher and Sakmann 1976) revolutionized the study of cellular physiology by providing a high-resolution method of observing the function of individual ionic channels in a variety of normal and pathological cell types. By

²Contributions by H. Gögelein.

the use of variations of the basic recording methodology, cellular function and regulation can be studied at a molecular level by observing currents through individual ionic channels (Liem et al. 1995; Sakmann and Neher 1995).

The most intriguing method is called the “on-cell” or “cell-attached” configuration, because ion channels can be recorded on an intact cell (Jackson 1993). This mode is well suited for investigation of ion channels that are activated by hormonal stimulation and triggered by intracellular second messengers.

Another versatile mode is the “cell-excised” configuration (Hamill 1993). It is obtained by suddenly removing the patch pipette from the cell, so that the membrane patch is pulled off the cell. This mode easily allows the investigator to expose the channel proteins to drugs by changing the bath solution. The single channel currents are recorded on a videotape and are analyzed off-line by a computer system. Various parameters are evaluated, such as the single channel conductance, open and closed times of the channel, and the open-state probability, which is the percentage of time the channel stays in its open state.

In addition to these modes, which enable the recording of single channel currents, it is also possible to measure the current flowing through the entire cell. This “whole-cell mode” is obtained by rupturing the membrane patch in the cell-attached mode (Hamill et al. 1981; Dietzel et al. 1993). This is achieved by applying suction to the interior of the patch pipette. The “whole-cell mode” allows not only the recording of electrical current, but also the measurement of cell potential. Moreover, the cell interior is dialyzed by the electrolyte solution contained in the patch pipette.

The fabrication of patch clamp pipettes has been described by Sakmann and Neher (1995) and Cavalieri et al. (1993).

Variations of the patch clamp technique have been used to study neurotransmitter transduction mechanisms (Smith 1995).

High throughput methods are required when developing drugs that work on ion channel function (Mathes 2003; Bennett and Guthrie 2003). Patch clamping suffers from low throughput, which is not acceptable for drug screening.

Fertig et al. (2002) and Brueggemann et al. (2004, 2006) presented nanopatch clamp technology, which is based on a planar, microstructured glass chip, which enables automatic whole-cell patch clamp experiments. Planar glass substrates containing a single microaperture produced by ion track etching are used

to record currents through ion channels in living mammalian cells.

Falconer et al. (2002) reported high throughput screening for ion channel modulators setting up a Beckman/Sagian core system to fully automate functional fluorescence-based assays that measure ion channel function. Voltage-sensitive fluorescent probes were applied and the activity of channels was measured using Aurora’s Voltage/Ion Probe Reader (VIPR). The system provides a platform for fully automated high throughput screening as well as pharmacological characterization of ion channel modulators.

Schroeder et al. (2003) described a high throughput electrophysiology measurement platform consisting of computer-controlled fluid handling, recording electronics, and processing tools capable of voltage clamp whole-cell recordings from thousands of individual cells per day. The system uses a planar, multiwell substrate (a PatchPlate). The system positions one cell into a hole separating two fluid compartments in each well of the substrate. Voltage control and current recordings from the cell membrane are made subsequent to gaining access to the cell interior by applying a permeabilizing agent to the intracellular side.

Willumsen’s group recommended ion channel screening with QPatch (Asmild et al. 2003; Kutchinsky et al. 2003; Krzywkowski et al. 2004). This system claims to allow fast and accurate electrophysiological characterization of ion channels, e. g., for determination of IC_{50} values for ion channel blockers. The system comprises 16 parallel patch clamp sites, each based on a silicon chip with a micro-etched patch clamp hole. Intra- and extra-cellular fluids are administered by laminar flow through integrated miniature flow channels.

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A.1.1.24.1

Patch Clamp Technique in Isolated Cardiac Myocytes

PURPOSE AND RATIONALE

The generation of an action potential in heart muscle cells depends on the opening and closing of ion-selective channels in the plasma membrane. The patch clamp technique enables the investigation of drug interactions with ion-channel-forming proteins at the molecular level.

PROCEDURE

Isolated cells from ventricular muscle of rat and guinea pig are prepared as described by Yazawa et al. (1990). Animals are sacrificed by cervical dislocation. Hearts are dissected and mounted on a Langendorff-type apparatus and perfused first with Tyrode solution (in mM: 143 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.25 NaH₂PO₄, 5 HEPES, pH adjusted to 7.4 with NaOH) at 37°C for 3 min at a hydrostatic pressure of 60–70 cmH₂O, then with nominally Ca²⁺-free Tyrode solution (no Ca²⁺ is added) for 5–7 min, and finally with nominally Ca²⁺-free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma, type I). After 15–20 min of collagenase treatment, the heart is now soft and is washed with storage solution (in mM: 70 KOH, 50 L-glutamic acid, 40 KCl, 20 taurine, 20 KH₂PO₄, 3 MgCl₂, 10 glucose, 10 HEPES, 0.5 EGTA, pH adjusted to 7.4 with KOH). The ventricles are cut into pieces (about 5 mm × 5 mm) and poured into a beaker. The myocytes are dispersed by gently shaking the beaker and filtration through a nylon mesh (365 μm). Then, the myocytes are washed twice by centrifugation at 600–1000 rpm (about 90 g) for 5 min and kept at room temperature. The rod shape of the cell and the clear striations of sarcomeres are important criteria for selecting viable cells for the assay. Experiments are performed at 35°C–37°C.

For investigation with the patch clamp technique (Neher and Sakmann 1976; Hamill et al. 1981), the isolated cells are placed into a thermostat-controlled chamber, mounted on the stage of an inverted microscope equipped with differential interference contrast optics. Under optical control (magnification 400×) a glass micropipette, having a tip opening of about 1 μm, is placed onto the cell. The patch pipettes are fabricated from borosilicate glass tubes (outer diameter 1.5 mm, inner diameter 0.9 mm) by means of an electrically heated puller. In order to prevent damage of the cell membrane, the tip of the micropipette is fire polished, by moving a heated platinum wire close to the tip. The patch pipette is filled with either high-NaCl or KCl solution and is mounted on a micro manipulator. A silver chloride wire connects the pipette solution to the head stage of an electronic amplifier. A second silver chloride wire is inserted into the bath and serves a ground electrode.

After establishing contact with the cell membrane, a slight negative pressure is applied to the inside of the patch pipette by means of a syringe. Consequently, a small patch of membrane is slightly pulled into the opening of the micro pipette and close contact

between the glass and membrane is formed, leading to an increase of the electrical input resistance into the giga-ohm range (about 10^{10} Ohm). This high input resistance enables the recording of small electrical currents in the range of picosiemens (10^{-12} S), which flow through channel-forming proteins situated in the membrane patch. The electrical current is driven by applying an electrical potential across the membrane patch, and/or by establishing an appropriated chemical gradient for the respective ion species.

The patch clamp methods allows one to investigate the interaction of drugs with all ion channels involved in the functioning of the heart muscle cell (K^+ , Na^+ , Ca^{2+} and eventually Cl^- channels). Moreover, the different types of K^+ channels existing in cardiomyocytes can be distinguished by their different single-channel characteristics or by appropriate voltage-pulse protocols in the whole-cell mode.

EVALUATION

Concentration–response curves of drugs which inhibit or activate ion channels can be recorded either at the single channel level or by measuring the whole-cell current. IC_{50} and EC_{50} values (50% inhibition or activation, respectively) can be obtained with both methods.

MODIFICATIONS OF THE METHOD

The patch clamp technique has been used for evaluation of anti-arrhythmic agents (Bennett et al. 1987; Anno and Hondeghem 1990; Gwilt et al. 1991).

Gögelein et al. (1998) used isolated ventricular myocytes from guinea pigs to study a cardioselective inhibitor of the ATP-sensitive potassium channel.

Multiple types of calcium channels have been identified by patch clamp experiments (Tsien et al. 1988).

The effects of potassium channel openers have been measured (Terzic et al. 1994).

Ryttsén et al. (2000) characterized electroporation of single NG108–15 cells with carbon-fiber microelectrodes by patch clamp recordings and fluorescence microscopy.

Monyer and Lambolez (1995) reviewed the molecular biology and physiology at the single cell level, discussing the value of the polymerase chain reaction at the single cell level and the use of patch pipettes for collecting the contents of a single cell on which the reverse transcription is performed.

The patch clamp technique was found to be very versatile in the investigation of ion channels in atrial myocytes, especially from dogs or humans. Cell were

obtained from atria either in sinus rhythm or in atrial fibrillation (reviewed in Bosch et al. 1999).

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A.1.1.24.2

Voltage Clamp Studies on Sodium Channels

PURPOSE AND RATIONALE

The epithelial Na^+ channel plays an important role in epithelial Na^+ absorption in the distal colon, urinary bladder, salivary and sweat ducts, respiratory tract and, most importantly, in the distal tubules of the kidney (Catterall 1986; Palmer 1992). Regulation of this epithelial Na^+ channel has a major impact on Na^+ balance, blood volume, and blood pressure. Inhibition of epithelial Na^+ channel expression is used for the treatment of hypertension (Endou and Hosoyamada 1995).

Busch et al. (1995) studied the blockade of epithelial Na^+ channels by triamterenes using two-microelectrode voltage clamp experiments in *Xenopus* oocytes expressing the three homologous subunits (α , β , and γ) of the rat epithelial Na^+ channel (rENaC).

PROCEDURE

Xenopus laevis oocytes are injected with the appropriate cRNA encoding for the α , β , and γ -subunits (Canessa et al. 1993) of the rat epithelial Na^+ channel (rENaC). The cRNA for the wild-type α -subunit and its deletion mutant $\Delta 278\text{--}273$ is always coinjected with an equal amount of β and γ -subunit cRNA (10 ng/oocyte).

Then, 2–8 days after cRNA injection, the two-microelectrode voltage clamp method is used to record currents from *Xenopus* oocytes. Recordings are performed at 22°C using a Geneclamp amplifier (Axon Instruments, Foster City, Calif., USA), and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). The ND 96 solution (control) contains (mM): NaCl 96, KCl 2, CaCl_2 1.8, MgCl_2 1, HEPES 5, pH 7.0. In some experiments, Na^+ is replaced by *N*-methyl-D-glucamine (NMDG) solution. The microelectrodes are filled with 3 M KCl solution and have resistances in the range 0.5–0.9 M Ω . Chemicals (e. g., triamterene as standard) are added at concentrations between 0.2 and 100 μM . The amplitude of the induced currents varies considerably, depending on the day of channel expression and the batch of oocytes. The mutant channel induces considerably smaller currents than the wild-type channel. The total Na^+ current amplitude is determined at least once for each experimental day by superfusion with NMDG solution, or with 3 μM or 5 μM amiloride solution at the beginning and at the end of each set of experiments.

EVALUATION

Data are presented as means \pm SEM. A paired Student's *t*-test is used. The level of statistical significance is set at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Nawada et al. (1995) studied the effects of a sodium, calcium, and potassium antagonistic agent on the sodium current by the whole cell voltage clamp technique (tip resistance = 5 M Ω [Na]_i and [Na]_o 10 mmol/l at 20°C) in guinea pig isolated ventricular cells.

Sunami and Hiraoka (1996) studied the mechanism of cardiac Na^+ channel block by a charged class I antiarrhythmic agent, in guinea pig ventricular myocytes

using patch clamp techniques in the whole-cell, cell-attached and inside-out configurations.

Erdő et al. (1996) compared the effects of *Vinca* derivatives on voltage-gated Na^+ channels in cultured cells from rat embryonic cerebral cortex. Effects on Na^+ currents were measured by applying voltage steps (20 ms duration) to -10 mV from a holding potential of -70 mV every 20 s. Steady-state inactivation curves were obtained by clamping the membrane at one of a series of 15-s prepulse potentials, followed 1 ms later by a 20-ms test pulse to -10 mV.

Ragsdal et al. (1993) examined the actions of a Na^+ channel blocker in whole-cell voltage clamp recordings from Chinese hamster ovary cells transfected with a cDNA encoding the rat brain type IIA Na^+ channel and from dissociated rat brain neurons.

Tagliatalata et al. (1996) studied cloned voltage-dependent Na^+ currents expressed in *Xenopus* oocytes upon injection of the cRNA encoding α -subunits from human and rat brain.

Wang et al. (1997) investigated pharmacological targeting of long QT mutant sodium channels.

Eller et al. (2000) measured the effects of a calcium antagonist on inward Na^+ currents (I_{Na}) in GH3-cells with the whole-cell configuration of the patch clamp technique. I_{Na} was recorded after depolarization from a holding potential of -80 mV to a test potential of $+5$ mV. Initial “tonic” block (resting state-dependent block) was defined as peak I_{Na} inhibition during the first pulse 2 min after drug application as compared with I_{Na} in the absence of drug. “Use- (frequency-) dependent” block of I_{Na} was measured during trains of 5- or 50-ms test pulses (3 Hz) applied from -80 mV to a test potential of $+5$ mV after a 2-min equilibrium period in the drug-containing solution. Use-dependent block was expressed as the percentage decrease of peak I_{Na} during the last pulse of the train as compared with I_{Na} during the first pulse.

Khalifa et al. (1999) characterized the effects of an antidepressant agent on the fast inward current (I_{Na}) in isolated guinea pig ventricular myocytes. Currents were recorded in the whole-cell configuration of the patch clamp technique in the presence of Ca^{2+} and K^+ channel blockers.

Haeseler et al. (1999) measured the effects of 4-chloro-*m*-cresol, a preservative added to a wide variety of drugs, on heterologously expressed wild-type, Paramyotonia congenita (R1448H) and hyperkalemic periodic paralysis (M1360V) mutant α -subunits of human muscle sodium channels using whole-cell and inside-out voltage clamp experiments.

Song et al. (2000) studied the effects of *N*-ethyl-maleimide, an alkylating agent to protein sulfhydryl groups, on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium channels in rat dorsal root neurons using the whole-cell configuration of the patch clamp technique. Rats at the age of 2–6 days were anesthetized with isoflurane and the spinal cord was removed and cut longitudinally. Dorsal root ganglia were plucked from the area between the vertebrae of the spinal column, and incubated in phosphate-buffered saline solution containing 2.5 mg/ml trypsin at 37°C for 30 min. After enzyme treatment, ganglia were rinsed with Dulbecco's Modified Eagle Medium supplemented with 10% horse serum. Single cells were mechanically dissociated by trituration with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated glass coverslips. Cells attached to the coverslips were transferred into a recording chamber on the stage of an inverted microscope. Ionic currents were recorded under voltage clamp conditions by the whole-cell patch clamp technique. The solution in the pipette contained (in mM): CsCl 125, NaF 20, HEPES 5, EGTA 5. The pH was adjusted to 7.2 with CsOH and the osmolarity was 279 mosmol/l on average. The external solution contained (in mM): NaCl 50, choline chloride 90, tetramethylammonium chloride 20, D-glucose 5, HEPES 5, MgCl₂ 1, CaCl₂ 1. Lanthanum (LaCl₃, 10 μM) was used to block calcium channel current. The solution was adjusted to pH 7.4 with tetramethylammonium hydroxide and the osmolarity was 304 mosmol/l on average. An Ag–AgCl pellet/3 M KCl-agar bridge was used for the reference electrode. Membrane currents were recorded using an Axopatch-1D amplifier. Signals were digitized by a 12-bit analog-to-digital interface, filtered with a low-pass Bessel filter at 5 kHz and sampled at 50 kHz using pCLAMP6 software (Axon Instruments) on an IBM-compatible PC. Series resistance was compensated 60%–70%. Capacitative and leakage currents were subtracted by using a P+P/4 procedure (Bezanilla and Armstrong 1977). The liquid junction potential between internal and external solutions was on average –1.7 mV. TTX (100 nM) was used to separate TTX-R sodium currents from TTX-S sodium currents. For the study of TTX-S sodium channels, cells that expressed only TTX-S sodium channels were used. TTX-S sodium channels were completely inactivated within 2 ms when currents were evoked by depolarizing steps to 0 mV, while TTX-R sodium channels persisted for more than 20 ms. The difference in kinetics was used to identify the type of sodium current.

Abriel et al. (2000) described the molecular pharmacology of the sodium channel mutation DI790G linked to long QT syndrome.

Makielski et al. (2003) showed that a ubiquitous splice variant and a common polymorphism affect heterologous expression of recombinant human SCN5AS heart sodium channels.

Viswanathan et al. (2001) studied gating mechanisms for flecainide action in *SCN5A*-linked arrhythmia syndromes.

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A.1.1.24.3

Voltage Clamp Studies on Potassium Channels

PURPOSE AND RATIONALE

Potassium channels represent a very large and diverse collection of membrane proteins which participate in important cellular functions regulating neuronal and cardiac electrical patterns, release of neurotransmitters, muscle contractility, hormone secretion, secretion of fluids, and modulation of signal transduction pathways. The main categories of potassium channels are gated by voltage or an increase of intracellular calcium concentration (Escande and Henry 1993; Kaczorowski and Garcia 1999; Alexander et al. 2001). For ATP-sensitive potassium channels see Sect. K.6.6.12.

The delayed outward potassium current in heart muscle cells of several species is made up of a **rapidly** (I_{K_r}) and a **slowly** (I_{K_s}) activating component (Sanguinetti and Jurkiewicz 1990; Wang et al. 1994; Gintant 1996; Lei and Brown 1996; Carmeliet and Mubagawa 1998). Several potent and selective blockers of the I_{K_r} channel have been shown to prolong the effective refractory period, but have a reverse rate-dependent activity with both normal and elevated extracellular potassium concentrations (Colatsky et al. 1990). Inhibitors of the slow component I_{K_s} were developed in order to circumvent the negative rate dependence of I_{K_r} channel blockers in the effective refractory period (Busch et al. 1996; Suessbrich et al. 1996, 1997; Bosch et al. 1998). Gögelein et al. (2000) studied the effects of a potent inhibitor of I_{K_s} channels in *Xenopus* oocytes and guinea pig ventricular myocytes.

PROCEDURE

Studies in *Xenopus* oocytes are performed with the two-microelectrode voltage clamp method. For isolation

of the oocytes, the toads are anesthetized using a 1 g/l solution of 3-aminobenzoic acid ethyl ester and placed on ice. A small incision is made to retrieve sacs of oocytes and is subsequently closed with absorbable surgical suture. On waking up, the toads are placed back into the aquarium. The ovaries are cut up into small pieces and the oocytes are washed in Ca^{2+} -free Or-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 5 mM HEPES; pH 7.4) and subsequently digested in Or-2 containing collagenase A (1 mg/ml, Worthington, type II) until follicles are not longer detectable on the oocyte's surface. The oocytes are stored at 18°C in recording solution ND-96 (NaCl 96 mM, KCl 2 mM, CaCl_2 1.8 mM, MgCl_2 1 mM, HEPES 5 mM, pH 7.4) with added sodium pyruvate (275 mg/l), theophylline (90 mg/l), and gentamicin (50 mg/l).

For electrophysiological recordings, the two-microelectrode voltage clamp configuration is used to record ion currents from *Xenopus* oocytes. Injection of cRNA is performed according to Methfessel et al. (1986) and Golding (1992). Oocytes are injected individually with cRNA encoding for the human protein minK, guinea pig Kir2.1, human *Herg*, human Kv1.5, mouse Kv1.3, or human HNC2. In the case of minK the functional potassium channel is a heteromultimer composed of the endogenous (*Xenopus*) KvLQT1 and the injected human minK. This heteromultimeric potassium current is then called I_{K_s} (Barhanin et al. 1996; Sanguinetti et al. 1996).

The electrophysiological recordings are performed at room temperature, using a Geneclamp amplifier (Axon Instruments), and MacLab D/A converter. The amplitudes of the recorded currents are measured at the end of the test voltage steps. To amplify the inward potassium current through Kir2.1 and HNC2, the external potassium concentration is raised to 10 mM KCl and the NaCl concentration lowered to 88 mM (ND-88). The microelectrodes are filled with 3 M KCl and have a resistance between 0.5 M Ω and 1 M Ω . During the recordings the oocytes are continuously perfused with ND-96 (or ND-88 in the case of Kir2.1 and HNC2). The test compounds are dissolved in dimethylsulfoxide (DMSO) and added to the buffer ND-96 or ND-88. The current amplitude is determined after 5 min of wash-in time.

For the isolation of *ventricular myocytes*, guinea pigs (weight about 400 g) or Sprague-Dawley rats of either sex are sacrificed by cervical dislocation. The hearts are dissected and perfused retrogradely via the aorta at 37°C: first with nominally Ca^{2+} -free Tyrode solution (in mmol/l): 143 NaCl, 5.4 KCl, 0.5 MgCl_2 , 0.25 NaH_2PO_4 , 10 glucose, 5 HEPES, pH 7.2, then

with Tyrode solution containing 20 mmol/l Ca^{2+} and 3 mg/ml collagenase type CLS II (Biochrom, Berlin Germany). After 5–10 min collagenase treatment the ventricles are cut up into small pieces in the storage solution (in mmol/l): 50 L-glutamic acid monopotassium salt, 40 KCl, 20 taurine, 20 KH_2PO_4 , 1 MgCl_2 , 10 glucose, 0.2 EGTA, pH 7.2. The myocytes are then dispersed by gentle shaking followed by filtration through a nylon mesh (365 μm). The cells are finally washed twice by centrifugation at 90 g for 5 min and kept in the storage solution at room temperature.

Whole-cell currents are recorded in the tight-seal whole-cell mode of the patch clamp technique, using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes are pulled from borosilicate glass capillaries (wall thickness 0.3 mm, outer diameter 1.5 mm) and their tips are fire-polished. Series resistance is in the range of 1–10 M Ω and 50% compensated by means of the EPC's compensation circuit.

The I_{Ks} , I_{Kr} , and I_{K1} currents in guinea pig ventricular myocytes are investigated. The voltage pulses for recording the current components are as follows: I_{Ks} current: holding potential -80 mV to -50 mV (200 ms) to $+60$ mV (3 s) to -40 mV (2 s) to -80 mV; I_{Kr} current: holding potential -80 mV to -50 mV (200 ms) to -10 mV (3 s) to -40 mV (2 s) to -80 mV. I_{Kr} is evaluated as the tail current evoked by a voltage pulse from -10 mV to -40 mV; I_{K1} : holding potential -80 mV to -120 mV (200 ms) to -80 mV. In order to suppress the L-type Ca^{2+} current, 5 mmol/l nifedipine is added to the bath solution.

EVALUATION

All average data are presented as means \pm SEM. Student's *t*-test is used to determine the significance of paired observations. Differences are considered as significant at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Using the whole-cell configuration of the patch clamp technique, Grissmer et al. (1994) analyzed the biophysical and pharmacological properties of five cloned voltage-gated K^+ channels stably expressed in mammalian cell lines.

Sanchez-Chapula (1999) studied the block of the transient outward K^+ channel (I_{to}) by disopyramide in isolated rat ventricular myocytes using whole-cell patch clamp techniques.

Using the patch clamp technique, Cao et al. (2001) investigated the effects of a centrally acting muscle re-

laxant and structurally related compounds on recombinant small-conductance Ca^{2+} -activated K^+ channels (rSK2 channels) in HEK mammalian cells.

Tagliatela et al. (2000) discussed the block of the K^+ channels encoded by the human *ether- \acute{a} -go-go-related* gene (HERG), termed $\text{K}_{\text{V}(\text{r})}$, which are the molecular determinants of the rapid component of the cardiac repolarizing current $I_{\text{K}(\text{Vr})}$, involved in the cardiotoxic potential and CNS effects of first-generation antihistamines and may be therapeutic targets for antiarrhythmic agents (Vandenberg et al. 2001; Zhou et al. 2005).

Chabbert et al. (2001) investigated the nature and electrophysiological properties of Ca^{2+} -independent depolarization-activated potassium currents in acutely isolated mouse vestibular neurons using the whole-cell configuration of the patch clamp technique. Three types of currents were identified.

Furthermore, Longobardo et al. (1998) studied the effects of a quaternary bupivacaine derivative on delayed rectifier K^+ currents stably expressed in *Ltk*⁻ cells using the whole-cell configuration of the patch clamp technique.

Moreno et al. (2003) studied the effects of a selective angiotensin II type 1 receptor antagonist on cloned potassium channels involved in human cardiac repolarization.

Sanchez-Chapula et al. (2002) investigated the voltage-dependent block of wild-type and mutant HERG K^+ channels by the antimalarial compound chloroquine.

Anson et al. (2004) published molecular and functional characterization of common polymorphism in HERG (KCNH2) potassium channels.

For more information on the evaluation of HERG potassium channels in safety pharmacology see Chap. I.D by Brian D. Guth.

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Studies on Kv1.5 Channel

PURPOSE AND RATIONALE

Treatment of atrial fibrillation/flutter with available potassium channel blockers (Class III antiarrhythmic agents which mainly block the delayed rectifier current I_{Kr}) is associated with ventricular proarrhythmia. Prolongation of ventricular repolarization leads to early after-depolarization from which torsades de pointes can evolve. Therefore, blockade of a cardiac current of exclusive relevance in the atria is highly desirable as it is expected to be devoid of ventricular proarrhythmic effects. The ultrarapid delayed rectifier potassium current (I_{Kur}) seems an ideal atrial antiarrhythmic target since it is found to contribute to the action potential in the atrium but not in the ventricle. The molecular correlate of the human cardiac ultrarapid delayed rectifier potassium current is the potassium channel Kv1.5, which therefore gained much interest (Li et al. 1996; Longobardo et al. 1998; Perchenet and Clément-Chomienne 2000; Caballero et al. 2000, 2001, 2004; Bachmann et al. 2001; Kobayashi et al. 2001; Matsuda et al. 2001; Choi et al. 2002; Moreno et al. 2003; Choe et al. 2003; Fedida et al. 2003; Godreau et al. 2002, 2003; Peukert et al. 2003, 2004; Plane et al. 2005).

For *in vivo* studies on atrial fibrillation see A.5.0.9, A.5.0.9.1, A.5.0.9.2, A.5.0.9.3.

Gögelein et al. (2004) studied the effects of the antiarrhythmic drug AVE0118 on cardiac ion channels.

PROCEDURE

Molecular Biology and Cell Culture

Human Kv1.5 cDNA was subcloned into the eukaryotic expression vectors pcDNA3.1 and pcDNA3.1/zeo (Invitrogen, Groningen, the Netherlands), cDNA encoding human Kv4.3 long (Kv4.3l; Dilks et al. 1999) was subcloned into pcDNA3.1, and the cDNA encoding human KChIP2 short (KChIP2.2; Decher et al. 2001) was subcloned into pcDNA3.1/zeo expression vector. Chinese hamster ovary (CHO) cells were transfected with either hKv1.5 or hKv4.3 and KChIP2.2 expression constructs. Transfection was carried out using lipofectamine (Life Technologies/Gibco BRL, Karlsruhe, Germany) according to the manufacturer's instructions. To boost Kv1.5 channel expression, CHO cells were consecutively transfected with both Kv1.5 expression constructs. Both hKv1.5 and hKv4.3+hKChIP2.2 were stably expressed in CHO cells, which were maintained in ISCOVE's medium (Biochrom KG, Berlin, Germany), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 350 µg/ml Zeocin (Invitrogen) and 400 µg/ml G418 (PAA Laboratories). HERG, the potassium channel underlying I_{Kr} currents in human hearts, was cloned and transfected into CHO cells as described previously (Rampe et al. 1997). Cells used for patch clamping were seeded on glass or plastic coverslips 12–36 h before use.

Northern blot analysis of Kv1.5 in the pig heart and cloning of pig Kv1.5

Polyadenylated RNA was isolated from pig cardiac tissues with the Oligotex mRNA purification kit (Qiagen) and 10 µg per tissue was resolved by denaturing formaldehyde electrophoresis and blotted on a positively charged nylon membrane. The membrane was hybridized with a DIG-labeled riboprobe (DIG RNA labeling kit, Roche) encompassing the entire coding sequence of human Kv1.5 and exposed on a Lumi-Imager (Roche). The pig Kv1.5 was cloned by 5'-rapid amplification and 3'-rapid amplification of cDNA ends (RACE) reactions. An adapter ligated, double-stranded cDNA library was prepared from pig heart mRNA with the Marathon cDNA amplification kit (Clontech). The 5'-RACE and 3'-RACE reactions were performed with oligonucleotide primers derived from a partial pig Kv1.5 nucleotide sequence (GenBank accession number AF348084). Overlapping cDNA clones were obtained by repeated reactions and the DNA sequence determined by automated DNA sequencing on both

strands (ABI 310, Perkin Elmer). A full-length cDNA clone was established by recombinant PCR. It encodes an open reading frame of 1,083 bp and a protein with 86% overall sequence similarity to the human Kv1.5 protein. The sequence of the pig Kv1.5 cDNA was submitted to GenBank (accession number: AY635585).

For *Xenopus* oocyte expression, cDNAs encoding Kv1.5, Kv4.3, and KChip2.2 were cloned into the oocyte expression vector pSGEM (Villmann et al. 1997), and capped cRNA was synthesized using the T7 mMessage mMachine kit (Ambion, Austin, Tex., USA).

Voltage-clamp experiments in *Xenopus* Oocytes

Handling and injection of *Xenopus* oocytes were performed according to Bachmann et al. (2001). Adult female *Xenopus laevis* frogs were anesthetized with 3-aminobenzoic acid ethyl ester solution (1 g/l) and intact ovary lobes were removed. The oocytes were defolliculated by treatment with 40 mg collagenase dissolved in 20 ml buffer (in mM: NaCl 82.5, KCl 2, MgCl₂ 1, HEPES 5, titrated to pH 7.5 with NaOH) for 120–150 min at 18°C. Oocytes were injected with 50 nl cRNA using a microinjector (World Precision Instruments, Sarasota, Fla., USA). Oocytes were stored under gentle shaking at 18°C in a buffer containing (in mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, Na-pyruvate 2.5, theophylline 0.5, gentamicin 50 µg/ml, titrated to pH 7.5 with NaOH. They were used for experiments 1–3 days after injection.

Two-electrode voltage clamp recordings were performed at room temperature in a medium containing (in mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, pH 7.5 with NaOH. Microelectrodes were pulled from filament borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). After filling with 3 M KCl, pipettes had a resistance of 0.3–1.3 MΩ. To activate hKv1.5 and hKv4.3 channels, oocytes were clamped from a holding potential of –80 mV to 40 mV for 500 ms. Data were recorded with a Turbo Tec 10CX amplifier (NPI, Tamm, Germany) using an ITC-16 interface (Instrutech Corporation, Long Island, USA) and the Pulse software (HEKA Elektronik, Lambrecht, Germany).

Patch Clamp Experiments with CHO Cells

Cells expressing Kv1.5 or Kv4.3 plus KChIP2.2 were assayed using the standard whole-cell patch clamp technique (Hamill et al. 1981). Cells were mechanically removed from the tissue culture flask and placed in a perfusion chamber with a solution containing (in

mM): NaCl 140, KCl 4.7, CaCl₂ 2, MgCl₂ 1.1, HEPES 10, pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries and heat polished. After filling with (in mM): NaCl 10, KCl 120, EGTA 1, HEPES 10, MgCl₂ 1.1 (pH 7.2 with potassium hydroxide, KOH), pipettes had resistances of 2–3 MΩ. Experiments were carried out at 36 ± 1°C. For the recording of hKv1.5, voltage pulses of 450 ms duration were applied from the holding potential of –30 mV to +20 mV at a frequency of 1 Hz. For recording of the hKv4.3+KChIP2.2, the holding potential was –50 mV and test pulses of 200 ms duration were applied to –10 mV at a frequency of 1 Hz. Data were recorded with an EPC-9 patch clamp amplifier (HEKA Elektronik) and the Pulse software (HEKA Elektronik) and stored on a PC for later analysis. Series resistance was in the range of 4–9 MΩ and was compensated by 80% by means of the EPC9's compensation circuit. The experiments were performed under continuous superfusion of the cells with solution heated to 36 ± 1°C.

HERG channel currents were recorded at room temperature using the whole-cell configuration of the patch clamp technique with an Axopatch 200B amplifier (Axon Instruments). Briefly, electrodes (3–6 MΩ resistance) were fashioned from TW150F glass capillary tubes (World Precision Instruments) and filled with pipette solution (in mM: potassium aspartate 120, KCl 20, Na₂ATP 4, HEPES 5, MgCl₂ 1, pH 7.2 adjusted with KOH). HERG currents were initiated by a positive voltage pulse (20 mV) followed by a negative pulse (–40 mV) and were recorded for off-line analyses. Once HERG current from a cell perfused with control external solution (in mM: NaCl 130, KCl 5, sodium acetate 2.8, MgCl₂ 1, HEPES 10, glucose 10, CaCl₂ 1 at pH 7.4 adjusted with NaOH) was stabilized, the cell was perfused with external solution containing the compound at a specific concentration for percentage inhibition. For each concentration from each cell, peak amplitude of the steady-state HERG tail current at –40 mV was measured. The peak amplitude for each concentration was compared with that for the control solution from the same cell and expressed as percent control.

Isolation of Porcine Atrial Myocytes

Male pigs weighing 15–30 kg of the German Landrace were anesthetized with pentobarbital exactly as described previously (Wirth and Knobloch 2001). After a left thoracotomy the lung was retracted, the pericardium incised and the heart was quickly removed and placed in oxygenated nominally Ca²⁺-free Tyrode solution containing (in mM): NaCl 143, KCl 5.4,

MgCl₂ 0.5, NaH₂PO₄ 0.25, HEPES 5 and glucose 10, pH adjusted to 7.2 with NaOH. The hearts were then mounted on a Langendorff apparatus and perfused via the left circumflex coronary artery with Tyrode solution (37°C) with constant pressure (80 cmH₂O). All coronary vessels descending to the ventricular walls were ligated, ensuring sufficient perfusion of the left atrium. When the atrium was clear of blood and contraction had ceased (≈5 min), perfusion was continued with the same Tyrode solution, which now contained 0.015 mM CaCl₂ and 0.03% collagenase (type CLS II, Biochrom KG, Berlin, Germany), until atrial tissue softened (≈20 min). Thereafter, left atrial tissue was cut into small pieces and mechanically dissociated by trituration. Cells were then washed with storage solution containing (in mM): L-glutamic acid 50, KCl 40, taurine 20, KH₂PO₄ 20, MgCl₂ 1, glucose 10, HEPES 10, EGTA 2 (pH 7.2 with KOH) and filtered through a nylon mesh. The isolated cells were kept at room temperature in the storage solution.

Isolation of Guinea Pig Ventricular Myocytes

Ventricular myocytes were isolated by enzymatic digestion according to Gögelein et al. (1998). Dunkin Hardy Pirbright White guinea pigs (weight about 400 g) were sacrificed by cervical dislocation. The hearts were dissected and perfused retrogradely via the aorta at 37°C with the same solutions as used for isolation of pig atrial myocytes.

Electrophysiological Recordings from Cardiac Myocytes

Whole-cell currents were recorded with an EPC-9 patch clamp amplifier (HEKA Elektronik) as described above for CHO cells. A small aliquot of cell-containing solution was placed in a perfusion chamber and after a brief period allowing for cell adhesion to the chamber, the cells were perfused with (in mM): NaCl 140, KCl 4.7, CaCl₂ 1.3, MgCl₂ 1.0, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries and heat polished. After filling with (in mM) KCl 130, MgCl₂ 1.2, HEPES 10, EGTA 10, K₂ATP 1, GTP 0.1, and phosphocreatine 5 (pH 7.2 with KOH) pipettes had a resistance of 2–3 MΩ. Series resistance was in the range of 6–12 MΩ and was compensated by 60%–70%. Offset voltages generated when the pipette was inserted in NaCl solution (1–5 mV) were zeroed before formation of the seal.

Effects of AVE0118 on the *I*_{KACH} were recorded from pig left atrial myocytes by applying voltage pulses of 500 ms duration from the holding potential of –80 mV to –100 mV. Carbachol (10 μM) was added

in order to evoke the I_{KACH} . After stabilization of the I_{KACH} (3 min), AVE0118 was added in increasing concentrations in the continuous presence of carbachol. The current was measured at the end of the pulse after 3 min of incubation at each concentration and inhibition of the carbachol-activated current was calculated. In some experiments, AVE0118 was washed out before application of the next higher concentration.

Also the L-type Ca^{2+} current was investigated in pig left atrial cells. In these experiments, KCl in the pipette was replaced by CsCl and voltage pulses of 300 ms duration were applied from the potential of -40 mV to 0 mV. Possible effects of AVE0118 on the currents I_{K1} , I_{Ks} , I_{Kr} and I_{KATP} were investigated in guinea pig ventricular myocytes. I_{K1} currents were recorded by a voltage step from -80 mV to -120 mV lasting for 200 ms. When I_{Ks} and I_{Kr} currents were recorded, $1 \mu\text{M}$ nisoldipine was added to the bath to block the L-type Ca^{2+} current. I_{Ks} was assessed by voltage pulse to $+60$ mV for 3 s, starting from -40 mV. I_{Kr} was evaluated as the tail current evoked by a voltage pulse from -10 mV to -40 mV. I_{KATP} was evoked by adding $1 \mu\text{M}$ rilmakalim (Krause et al. 1995) to the bath and by applying voltage ramps from -130 mV to $+80$ mV for 500 ms. The rilmakalim-activated current was recorded at the potential 0 mV. All patch clamp experiments were performed under continuous superfusion of the cells with solution heated to $36 \pm 1^\circ\text{C}$.

EVALUATION

All averaged data are presented as the mean \pm SEM. The Student's t -test was used to determine the significance of paired or unpaired observations. Differences were considered significant at $P < 0.05$. The values for half-maximal inhibition (IC_{50}) and the Hill coefficient were calculated by fitting the data points of the concentration–response curves to the logistic function:

$$f(x) = (a - d) / [1 + (x/c)^n] + d$$

where a represents the plateau value at low drug concentration and d the plateau value at high drug concentration; c represents the IC_{50} value and n the Hill coefficient. The curve-fitting and the Student's t -test were performed with the computer program *Sigma-Plot* 5.0.

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A.1.1.24.4

Voltage Clamp Studies on Calcium Channels

PURPOSE AND RATIONALE

Calcium influx through voltage-gated Ca^{2+} channels mediates a range of cytoplasmic responses, including muscle contraction, release of neurotransmitters, Ca^{2+} dependent gene transcription and the regulation of neuronal excitability has been reviewed by several authors (Augustine et al. 1987; Bean 1989; Miller 1987; Zamponi 1997; Snutch et al. 2001). In addition to their normal physiological function, Ca^{2+} channels as calcium antagonists are also implicated in a number of human disorders (see also A.4.0.1).

Using patch clamp techniques, the structure and regulation of voltage-gated Ca^{2+} channels has been studied by many authors (Sculptoreanu et al. 1993; Peterson et al. 1997; Catterall 2000).

Berjukow et al. (2000) analyzed the role of the inactivated channel conformation in molecular mechanism of Ca^{2+} channel block by a dihydropyridine derivative

in L-type channel constructs and mutants in *Xenopus* oocytes and described the electrophysiological evaluation.

PROCEDURE

Inward barium currents (I_{Ba}) are studied with two microelectrode voltage-clamp of *Xenopus* oocytes 2–7 days after microinjection of approximately equimolar cRNA mixtures of constructs of L-channel mutants. All experiments are carried out at room temperature in a bath solution with the following composition: 40 mM $Ba(OH)_2$, 50 mM NaOH, 5 mM HEPES, 2 mM CsOH (pH adjusted to 7.4 with methanesulfonic acid). Voltage recording and current injecting microelectrodes are filled with 2.8 M CsCl, 0.2 M CsOH, 10 mM EGTA, 10 mM HEPES (pH 7.4) with resistances of 0.3–2 M Ω . Resting channel block is estimated as peak I_{Ba} inhibition during 100-ms test pulses from -80 to 20 mV at a frequency of 0.033 Hz until steady state is reached. The dose response curves of I_{Ba} inhibition were fitted using the Hill equation:

$$\frac{I_{Ba, \text{drug}}}{I_{Ba, \text{control}}} (\%) = \frac{100 - A}{1 + \left(\frac{C}{IC_{50}}\right)^{nH}} + A$$

where IC_{50} is the concentration at which I_{Ba} inhibition is half-maximal, C is the applied drug concentration, A is the fraction of I_{Ba} that is not blocked, and nH is the Hill coefficient.

Recovery from inactivation is studied at a holding potential of -80 mV after depolarizing Ca^{2+} channels during a 3-s prepulse to 20 mV by applying 30-ms test pulses (to 20 mV) at various time intervals after the conditioning prepulse. Peak I_{Ba} values are normalized to the peak current measured during the prepulse, and the time course of I_{Ba} recovery from inactivation is fitted to a mono- or biexponential function

$$I_{Ba, \text{recovery}} = A \times \exp\left(\frac{-t}{\tau_{\text{fast}}}\right) + B \times \exp\left(\frac{-t}{\tau_{\text{slow}}}\right) + C$$

Voltage dependence of inactivation under quasi-steady state conditions is measured using a multi step protocol to account for run-down (less than 10%). A control test pulse (50 ms to 20 mV) is followed by a 1.5-s step to -100 mV followed by a 30-s conditioning step, a 4-ms step to -100 mV, and a subsequent test pulse to 20 mV (corresponding to the peak potential of the I-V curves).

Inactivation during the 30 s conditioning pulse is calculated as follows.

$$I_{Ba, \text{inactivation}} = \frac{I_{Ba, \text{test}}(20 \text{ mV})}{I_{Ba, \text{control}}(20 \text{ mV})}$$

The pulse sequence is applied every 3 min from a holding potential of -100 mV. Inactivation curves are drawn according to the following Boltzmann equation.

$$I_{\text{Ba, inactivation}} = I_{\text{SS}} + (1 - I_{\text{SS}}) \left(1 + \exp \left(\frac{V - V_{0.5}}{k} \right) \right)$$

where V is the membrane potential, $V_{0.5}$ is the mid-point voltage, k is the slope factor, and I_{SS} is the fraction of non inactivating current.

Steady state inactivation of the mutant channels at -80 mV is estimated by shifting the membrane holding potential from -80 to -100 mV. Subsequent monitoring of the corresponding changes in I_{Ba} amplitudes until steady state reveals the fraction of Ca^{2+} channels in the inactivated state at -80 mV. Steady state inactivation of different L-type channel constructs at -30 mV is estimated by fitting time course of current inactivation to a biexponential function.

The I_{Ba} inactivation time constants are estimated by fitting the I_{Ba} decay to a mono- or biexponential function.

EVALUATION

Data are given as the means \pm SE. Statistical significance is calculated according to Student's unpaired t -test.

MODIFICATIONS OF THE METHOD

Besides *Xenopus* oocytes (Waard and Campell 1995; Hering et al. 1997; Kraus et al. 1998), several other cell types and constructs, such as CHO cells (Sculptoreanu et al. 1993; Stephens et al. 1997), HEK293 (human embryonic kidney) cells (Lacinová et al. 1999), tsA-201 cells, a subclone of HEK293, (Peterson et al. 1997; McHugh et al. 2000), cardiac myocytes from rats (Scamps et al. 1990; Tohse et al. 1992; Gomez et al. 1994) and rabbits (Xu et al. 2000), isolated atrial myocytes from failing and non-failing human hearts (Cheng et al. 1996), skeletal muscle myotubes from mice and rabbits, (Johnson et al. 1994), myocytes of guinea pig mesentery artery (Morita et al. 1999), dendrites from rat pyramidal and olfactory bulb neurons (Markram and Sakmann 1994; Stuart and Spruston 1995; Koester and Sakmann 1998; Margie et al. 2001), rat amygdala neurons (Foehring and Srcoggs 1994; Young et al. 2001) were used to study the function of calcium channels.

Using the whole-cell variation of the patch-clamp technique, Yang et al. (2000) studied cellular T-type and L-type calcium channel currents in mouse neuroblastoma N1E115 cells. The cells were cultured in

Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 3–4 days. After mechanical agitation, 3×10^4 cells were replanted in 35-mm tissue culture dishes containing 4 ml of bath solution. After cell attachment, the dish was mounted on the stage of an inverted phase-contrast microscope for Ca^{2+} channel current recording. These cells expressed predominantly T channel currents. In experiments where L channels were specifically sought, the cells were grown and maintained at confluence for 3–4 weeks under the same culture conditions with the addition of 2% dimethylsulfoxide (Narahash et al. 1987). Three to 5 days before use, the cells were replanted with the same medium. These cells expressed predominantly L channel currents. A small number of these cells also expressed T channel currents. Hence, cells were selected so that at a holding potential of -40 mV, the T channel component was very small and the inward current measured was conducted predominantly by L channels.

By using whole-cell and perforated patch-clamp techniques, Wu et al. (2000) showed that mifrabidile, a non-dihydropyridine compound, has an inhibitory effect on both T- and L-type Ca^{2+} currents in pancreatic β -cells.

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A.1.1.24.5

Patch Clamp Studies on Chloride Channels

PURPOSE AND RATIONALE

Cl^- channels are a large, ubiquitous and highly diverse group of ion channels involved in many physiological key processes including: regulation of electrical excitability; muscle contraction; secretion; and sensory signal transduction. Cl^- channels belong to several distinct families characterized in detail: voltage-gated Cl^- channels, the cAMP-regulated channel CFTR (cystic fibrosis transmembrane conductance regulator), ligand-gated Cl^- channels that open upon binding to the neurotransmitters GABA or glycine, and Cl^- channels that are regulated by the cytosolic Ca^{2+} concentration (Jentsch and Günther 1997; Frings et al. 2000).

Cliff and Frizel (1990) studied the cAMP- and Ca^{2+} -activated secretory Cl^- conductances in the Cl^- secreting colonic tumor epithelial cell line T84 using the whole-cell voltage-clamp technique.

PROCEDURE

T84 cells are used 1–3 days after plating on collagen-coating coverslips. The cells are maintained at 37°C. At this temperature, the responsiveness of the cells to secretagogues, particularly to cAMP-dependent agonists, is improved. Increases in Cl^- and K^+ conductances are the major electrical events during stimulation of Cl^- secretion. Accordingly, bath-pipette ion gradients are chosen so that transmembrane Cl^- and K^+ currents can be monitored independently at clamp voltages equal to the reversal potentials of these ions. The pipette solution is: 115 mM KCl, 25 mM N-methyl-D-glucamine (NMDG) glutamate, 0.5 mM

EGTA, 0.19 mM CaCl₂, 2 mM MgCl₂, 2 mM Na₂ATP, 0.05 mM Na₃GPT, 5 mM HEPES, pH 7.2. The bath solution is: 115 mM NaCl, 40 mM NMDG glutamate, 5 mM potassium glutamate, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.2. Bath Na⁺ and Cl⁻ concentrations are reduced by substituting NMDG chloride or sodium glutamate for NaCl. When Na⁺ and K⁺ free solutions are used, Na⁺ and K⁺ are replaced by NMDG⁺, and Cl⁻ is reduced by replacing Cl⁻ by glutamate.

During whole-cell recording, the membrane potential is clamped alternately to three different voltages, each for 500-msec duration. Computer-controlled voltage-clamp protocols are used to generate current-voltage (I-V) relations when the transmembrane currents are relatively stable by stepping the clamp voltage between -100 and +100 mV at 20 mV intervals.

Test drugs (e. g., 8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate, A23187, forskolin, or ionomycin) are solubilized in stock solutions (ethanol of DMSO) and diluted.

EVALUATION

Instantaneous relations are constructed from currents recorded 6 msec after a voltage step.

MODIFICATIONS OF THE METHOD

Maertens et al. (2000) used the whole-cell patch-clamp technique to study the effect of an antimalarial drug on the volume-regulated anion channel (VRAC) in cultured bovine pulmonary artery endothelial cells. They also examined the effects on other Cl⁻ channels, i. e., the Ca²⁺ activated Cl⁻ channel and the cystic fibrosis transmembrane conductance regulator to assess the specificity for VRAC.

Pusch et al. (2000) characterized chloride channels belonging to the CIC family. Chiral clofibrac acid derivatives were tested on the human CIC-1 channel, a skeletal muscle chloride channel, after heterologous expression in *Xenopus laevis* oocytes by means of two microelectrode voltage clamp recordings.

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A.1.1.25

Inhibition of Hyperpolarization-Activated Channels

PURPOSE AND RATIONALE

The hyperpolarization-activated cation currents (termed *I_f*, *I_h*, or *I_q*) play a key role in the initiation of cardiac and neuronal pacemaker depolarizations. Unlike most voltage-gated channels, they are activated by hyperpolarizing voltage steps to potentials negative to -60 mV, near the resting potential of most cells. This property earned them the designation of *I_f* for “funny” or *I_q* for “queer.” The funny current, or pacemaker (*I_f*) current, was first described in cardiac pacemaker cells of the mammalian sino-atrial node as a current that slowly activates on hyperpolarization at voltages in the diastolic voltage range, and contributes to the generation of cardiac rhythmic activity and to its control by sympathetic and parasympathetic innervations (DiFrancesco et al. 1986; Accili et al. 1997, 2002; Robinson and Siegelbaum 2003; Baruscotti et al. 2005). In sino-atrial cells, f-channels are modulated by cAMP independently of phosphorylation, through a mechanism involving direct interaction of cAMP with the intracellular side of the channels (DiFrancesco and Tortora 1991; Bois et al. 1996). A significant advancement in the study of molecular properties of pacemaker channels was achieved when a new family of channels was cloned, the HCN (hyperpolarization-activated, cyclic nucleotide gated) channels (Ishii et al. 1999; Kaupp and Seifert 2001; Biel et al. 2002; Macri et al. 2002). The HCN family is related to the cyclic nucleotide-gated channel and *eag* potassium channel family and belongs to the superfamily of voltage-gated cation channels. HCN channels are characterized by six membrane-spanning segments (S1–S6) including voltage-sensing (S4) and pore (between S5 and S6) regions. In the C-terminal region they contain a consensus sequence for binding of cyclic nucleotides. In the heart, neurotransmitter-induced control of cardiac rhythm is mediated by *I_f* through its second-messenger cAMP, whose synthesis is stimulated and inhibited by β-adrenoceptor and muscarinic agonists, respectively.

Inhibition of the *I_f* channel was recommended for induction of bradycardia and treatment of coronary disease (Thollon et al. 1994, 1997; Simon et al. 1995; Bois et al. 1996; Deplon et al. 1996; Acilli et al. 1997;

Rocchetti et al. 1999; Monnet et al. 2001, 2004; Bucchi et al. 2002; Cerbai et al. 2003; Rigg et al. 2003; Vilaine et al. 2003; Albaladejo et al. 2004; Colin et al. 2004; DiFrancesco and Camm 2004; Moreno et al. 2004; Mulder et al. 2004; Vilaine 2004; Chatelier et al. 2005; Leoni et al. 2005; Romanelli et al. 2005; Schipke et al. 2006).

Romanelli et al. (2005) reported the design, synthesis and preliminary biological evaluation of zatebradine analogs as potential blockers of hyperpolarization-activated current and Chatelier et al. (2005) described that a calmodulin antagonist directly inhibits f-type current in rabbit sino-atrial cells.

PROCEDURE

Sino-atrial Cell Isolation

Sino-atrial node myocytes of the rabbit were isolated (DiFrancesco et al. 1986). Cells were allowed to settle in Petri dishes, and were superfused with normal Tyrode solution containing (mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, D-glucose 5.5, Hepes-NaOH 5; pH 7.4.

Electrophysiology

In macro-patch experiments the temperature was kept at 27°C–28°C and the patch pipette solution contained (mM): NaCl 70, KCl 70, CaCl₂ 1.8, MgCl₂ 1, BaCl₂ 1, MnCl₂ 2, Hepes-KOH 5; pH 7.4. The control solution perfusing the intracellular side of the membrane patches contained (mM): potassium aspartate 130, NaCl 10, CaCl₂ 2, EGTA 5, Hepes-KOH 10; pH 7.2, $pCa = 7$. In some experiments, the calcium concentration of the bath solution was reduced to 0.1 nM according to the calculation of Fabiato and Fabiato (1979) and the correction of Tsien and Rink (1980).

Macro-patches containing hundreds of f-channels were formed using a large-tipped pipette (0.5 to 2 M Ω) (DiFrancesco and Tortora 1991). The test compound or calmodulin (Calbiochem) was dissolved in either distilled water and ethanol (50/50) or distilled water, respectively, divided into aliquots, and stored at –20°C until use. Ethanol was added to control solutions at the same concentration used in test solutions (lower than 0.1%).

EVALUATION

The time course of macro-patch I_f under the influence of the modifying compounds was recorded by applying hyperpolarizing steps of 3 s duration at a frequency of 1/15 Hz. At steady-state, the voltage dependence of I_f was described by the equation: $I_f(E) = g_f(E) \cdot (E - vE_f) = g_{fmax} \cdot y_{\infty}(E) \cdot (E - E_f)$,

where g_f is the conductance, g_{fmax} the fully activated conductance, $y_{\infty}(E)$ the steady-state activation parameter and E_f the reversal potential (DiFrancesco and Noble 1985). Steady-state current/voltage (I/V) curves were measured by applying 1-min-long hyperpolarizing voltage ramps with a rate of –115 mV/min from a holding potential of –35 mV. Conductance–voltage (g_f/E) relations were then obtained from the above equation as ratios between steady-state I/V curves (i_f/E) and $E - E_f$, where E_f was set to –12.24 mV (DiFrancesco and Mangoni 1994). Conductance curves were fitted by Boltzmann function: $g_f(E) = g_{fmax} \cdot y_{\infty}(E) = g_{fmax} \cdot 1/[1 + \exp(E - E_{1/2})/p]$ where $E_{1/2}$ is the half-maximal voltage of activation and p is the inverse-slope factor. This allowed estimation of the shifts of the voltage dependence of conductance (i.e., of the activation parameter y_{∞}) measured as changes in $E_{1/2}$. Shifts of the I_f activation curve caused by cAMP were also determined by a quicker method not requiring measurement of the conductance–voltage relation (Accili and DiFrancesco 1996). Shifts were obtained by applying hyperpolarizing steps from –35 mV to near the mid-point of the I_f activation curve and adjusting the holding potential (–35 mV in the control solution) until the cAMP-induced change in I_f was compensated and the control I_f magnitude fully restored. Since the compensation involved a change of the test voltage (from E to $E + s_m$, where s_m is the measured displacement of the holding potential in mV), a correction was introduced to obtain the shift of the activation curve (s , mV), according to the relation: $s = s_m \cdot [+(y_{\infty} / (dy_{\infty} / dE)) / (E - E_f)]$.

When comparing different sets of data, statistical analysis was performed with either the Student's t -test or analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant. Statistical data were given as mean \pm SEM values.

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A.1.1.26 Measurement of Cytosolic Calcium with Fluorescent Indicators

PURPOSE AND RATIONALE

Intracellular free Ca-concentration can be measured in cultured endothelial cells with a fluorometric methods (Tsien et al. 1982; Grynkiewicz et al. 1985; Lückhoff et al. 1988; Busse and Lamontagne 1991; Hock et al. 1991).

PROCEDURE

Cultured endothelial cells from the pig are seeded on quartz coverslips and grown to confluence. The cells

are loaded with the fluorescent probe indo-1 by incubation with 2 μmol indo-1/AM and 0.025% Pluronic F-127, a non-ionic detergent. Thereafter, the coverslips are washed and transferred to cuvettes, filled with HEPES buffer.

EVALUATION

Fluorescence is recorded in a temperature controlled (37°C) spectrofluorophotometer (exciting wavelength 350 nm, emission wavelength simultaneously measured at 400 and 450 nm).

MODIFICATIONS OF THE METHOD

Lee et al. (1987) measured cytosolic calcium transients from the beating rabbit heart using indo-1 AM as indicator.

Yangisawa et al. (1989) measured intracellular Ca^{2+} concentrations in coronary arterial smooth muscle of dogs with fura-2.

Makujina et al. (1995) measured intracellular calcium by fura-2 fluorescence simultaneously with tension in everted rings of porcine coronary artery denuded of endothelium.

Hayashi and Miyata (1994) described the properties of the commonly used fluorescent indicators for intracellular calcium: Fura-2, Indo-1, and Fluo-3.

Monteith et al. (1994) studied the Ca^{2+} pump-mediated efflux in vascular in spontaneously hypertensive rats.

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A.1.1.27

Measurement of Contractile Force of Isolated Cardiac Myocytes

PURPOSE AND RATIONALE

Eschenhagen et al. (1997) developed a method for culturing embryonic cardiomyocytes in a collagen matrix to produce a coherently contracting 3-dimensional model heart tissue that allows direct measurement of isometric contractile force.

PROCEDURE

Ventricles from 9–11 day incubated chicken embryos (Cavanaugh 1955) are minced in Dulbecco's minimal essential medium (DMEM), washed once with 0.25% trypsin/0.1% EDTA in phosphate buffered saline (PBS), pH 7.45, and then digested in fresh trypsin/EDTA for 15 min at 37°C. The supernatant is discarded and the pellet is subjected to digestion with 0.1% collagenase (144 U/mg) in PBS, pH 7.45, for 30 min at 37°C. This supernatant is discarded and the pellet digested further with several cycles of collagenase for 10–20 min each until the pellet is completely digested. DNase I (40 μl , 1 mg/ml in PBS) is added between cycles depending on the presence of viscous DNA. The isolated cells are kept in Petri dishes in DMEM supplemented with 15% heat-inactivated fetal calf serum in the CO_2 incubator. After completion of the digestion, the cells are incubated for another 30–60 min in the CO_2 incubator (preplating). The cell suspension is centrifuged at 250 rpm (12 g). The pellet is resuspended in 10 ml culture medium (DMEM, 10% inactivated horse serum, 2% chicken embryo extract (Gibco BRL), 2 mmol/l glutamine, 10 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin G, recentrifuged at 250 rpm, and finally resuspended in culture medium at $2\text{--}3 \times 10^6$ cells per ml.

For casting cardiomyocyte-populated collagen gels, strips of Velcro are glued with silicone rubber to glass

tubes (13 mm length, 3 mm outer diameter, 2 mm inner diameter). Pairs of Velcro-coated tubes, kept at a fixed distance by a stainless steel wire spacer, are placed in rectangular wells (15 × 17 × 4 mm) cut into a layer of silicone rubber in a 100 mm polymethylenepentene Petri dish. This assembly is autoclaved before use. For each gel, 1 ml of an ice-cold collagen/cell mixture is poured into each well between the Velcro-coated glass tubes. This mixture has the same composition as the culture medium and contains in addition to 1 mg neutralized collagen I from rat tail (Upstate Biotechnology, Inc.), 1×10^6 cardiomyocytes, the acetic acid in the collagen solution, and the NaOH to neutralize it. The mixture is allowed to gel at 37°C for 60 min before culture medium is added to the dish. Medium changes are performed after overnight and then every other day.

After 6–11 days in culture, the gels are removed from the culture dish, the spacers are withdrawn, and one of the glass tubes is mounted on a fixed electrode; the other tube is connected by an inelastic silk string to an isometric force transducer attached to a Wekagraph thermal array recorder (Föhr Instruments, Heidelberg, Germany). The preparation is adjusted to its original (spacer) length before it is immersed in a conventional water bath filled with modified Tyrode's solution maintained at 35°C and continuously gassed with 95% O₂ and 5% CO₂.

After a 30–60 min equilibration period without pacing, force and frequency reaches a stable value. Gels are then electrically stimulated with rectangular pulses (10 ms, 20–40 V) at a standard frequency of 1.5 Hz. Preload is stepwise adjusted to L_{\max} , the length at which the preparation develops maximal force. Cumulative doses of inotropic compounds, e.g. isoprenaline or forskoline, are added. All gels are exposed to a concentration-response curve for calcium (1.8–12.6 mmol/l) and one or two additional inotropic stimuli.

EVALUATION

All values are presented as arithmetic means \pm SEM. Student's *t*-test for paired observations is used to compare force of contraction, resting tension, or beating frequency before and after other interventions.

MODIFICATIONS OF THE METHOD

Ferrara et al. (1997) studied the role of Gi-proteins and β -adrenoceptors in the age-related decline of contraction in guinea-pig ventricular myocytes. The isolated myocytes were placed in Krebs-Henseleit solution in a Perspex chamber on the stage of a Zeiss IM inverted microscope and superfused with Krebs-Henseleit so-

lution containing 1 mmol/l Ca²⁺ at 2 ml/min and 32°C. Cells were selected using the following criteria: rod shaped, without sarcolemmal blebs, no spontaneous contractions, stable baseline contraction to electrical stimulation at 0.5 Hz and sarcomere length not shorter than 1.67 μ m. The image of the cells was displayed on a TV monitor and the length change measured with a video motion detector. Contraction amplitude and velocity was expressed as change in sarcomere length, calculated from the change in length of the myocyte and its original sarcomere length.

Using a similar technique, Harding et al. (1988) studied contractile responses of isolated rat and rabbit myocytes to isoproterenol and calcium, and Harding et al. (1992) isolated ventricular myocytes from failing and non-failing human heart.

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A.1.1.28

Adrenomedullin

A.1.1.28.1

General Considerations

Adrenomedullin is a 52-amino acid peptide originally discovered in human adrenal pheochromocytoma by monitoring the elevating activity of platelet cAMP (Kitamura et al. 1993). Molecular cloning of rat adrenomedullin was reported by Sakata et al. (1993). The genomic structure of human adrenomedullin gene was reported by Ishimitsu et al. (1994). Adrenomedullin and **proadrenomedullin N-terminal 20 peptide (PAMP)** which are both hypotensive and bronchodilating, are derived from **preproadrenomedullin** (Kanazawa et al. 1995; Iwasaki et al.

1996; Shimosawa and Fujita 1996; Hinson et al. 1998; Samson 1998; Autelitano and Tang 1999; Jimenez et al. 1999; Lopez et al. 1999; Tajima et al. 1999).

Adrenomedullin is found ubiquitously in tissues and organs, especially in cardiovascular tissues and in the kidney, lung, brain and endocrine glands (Wimalawansa 1996; Van Rossum et al. 1997; Eto et al. 1999; Jougasaki and Burnett 2000; Kitamura et al. 2000). The main biological effect is vasodilatation (Ishiyama et al. 1993; Nikitenko et al. 2002). A hypotensive effect has been found in rats (Khan 1997), rabbits (Fukuhara et al. 1995), and man (Lainchbury et al. 1997). Adrenomedullin belongs to the calcitonin gene-related peptide/calcitonin peptide family as it shares approximately 25% homology with calcitonin gene-related peptide (Kitamura et al. 1993). Several pharmacological studies are related to the vasodilating effect of adrenomedullin, e.g., in mouse aorta (Ashton et al. 2000), in the mesenteric vascular bed (see A.8.2.5), (Santiago et al. 1995), in the hind limb vascular bed (see A.8.2.1), (Santiago et al. 1994; Champion et al. 1996, 1997), in the pulmonary vascular bed (see A.8.2.6), (DeWitt et al. 1994; Lipp-ton et al. 1994; Heaton et al. 1995; Nossaman et al. 1995), on cerebral blood flow in dogs (Baskaya et al. 1995) and in cats (Takao et al. (1999), on renal hemodynamics in dogs (see A.8.2.3) (Ebara et al. 1994; Yukawa 1998), or on vasodilation in perfused rat kidneys (Hayakawa et al. 1999). Intravenous infusion of adrenomedullin exerted diuresis and natriuresis without major changes in blood pressure and produced beneficial hemodynamic and renal vasodilator effects in rats with compensated heart failure (Vari et al. 1996; Nagaya et al. 1999). In isolated perfused, paced rat heart preparations, adrenomedullin showed a dose-dependent inotropic effect (Szokodi et al. 1998). Pulmonary vasodilator responses and vasorelaxant effects in isolated pulmonary artery rings were found by Gumusel et al. (1998). Adrenomedullin is a growth-promoting factor for cultured vascular smooth muscle cells (Iwasaki et al. 1998) and fibroblasts (Isumi et al. 1998).

Willenbrock et al. (1999) showed a beneficial effect of adrenomedullin on renal function in rats with aorto-caval shunt.

Adrenomedullin inhibits gastric secretion in rats with chronic gastric fistula (see J.3.1.3), (Rossowski et al. 1997) and inhibits reserpine-induced gastric lesions in rats (Clementi et al. 1998). Tsuchida et al. (1999) found an inhibition of cholecystokinin-stimulated amylase secretion by adrenomedullin in rat pancreatic acini.

Rademaker et al. (2003) discussed the role of adrenomedullin in the pathophysiology of heart failure.

Lewis et al. (1998) described a specific and sensitive radioimmunoassay for human adrenomedullin.

Ohta et al. (1999) developed an one-step direct assay for adrenomedullin with monoclonal antibodies.

N-terminal fragments of adrenomedullin show vasopressor activities (Watanabe et al. 1996).

Adrenotensin, an other adrenomedullin gene product, contract in an endothelium-dependent manner pulmonary blood vessels (Gumusel et al. 1996).

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A.1.1.28.2

Receptor Binding of Adrenomedullin

PURPOSE AND RATIONALE

Muff et al. (1995), Poyer (1997) reviewed the binding characteristics of the structurally related hormones calcitonin, calcitonin gene-related peptide, amylin, and adrenomedullin. Vine et al. (1996) compared *in vitro* binding of adrenomedullin, calcitonin gene-related peptide and amylin.

Specific adrenomedullin binding sites were described in human brain (Sone et al. 1997), in the rat spinal cord (Owji et al. 1996), in cultured brain cells (Zimmermann et al. 1996), and in cultured rat mesangial cells (Osajima et al. 1996).

PROCEDURE

Human brain is obtained at autopsy. For preparation of membranes, tissues are homogenized in ice-cold 50 mM HEPES buffer, pH 7.6; containing 0.25 M sucrose, 10 µg/ml soybean trypsin inhibitor, 0.5 µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.5 µg/ml antipain, 0.1 mg/ml benzamidine, 0.1 mg/ml bacitracin, and 30 µg/ml aprotinin. The homogenates are centrifuged at 1500 g for 20 min at 4°C. The pellets are resuspended in 10 vol of the above buffer without sucrose and centrifuged at 100,000 g for 1 h at 4°C. The final pellets are resuspended to a concentration of 2–10 mg protein/ml, aliquoted, and stored at –80°C.

For the receptor binding assay, brain membranes (100 µg protein) are incubated at 4°C in 0.5 ml binding buffer (20 mM HEPES buffer, pH 7.4, containing 5 mM MgCl₂, 10 mM NaCl, 4 mM KCl, 1 mM EDTA, and 0.3% BSA) containing 0.3 nM [¹²⁵I]-human adrenomedullin in siliconized microcentrifuge tubes. Pellets are washed with 0.5 ml binding buffer at 4°C and counted in a γ-counter.

EVALUATION

Nonspecific binding is determined in the presence of 200 nM unlabeled human adrenomedullin. Specific

binding is defined as total binding minus nonspecific binding. Data are calculated as mean ± SEM.

MODIFICATIONS OF THE METHOD

Eguchi et al. (1994) studied the binding of human adrenomedullin and analogs and the adenylate cyclase activity in cultured rat vascular smooth muscle cells.

Zimmermann et al. (1995) showed that adrenomedullin and calcitonin gene-related peptide interact with the same receptor in cultured human neuroblastoma SK-N-MC cells.

Moody et al. (1997) investigated the binding affinity of adrenomedullin in C6 glioma cells.

Findings of Belloni et al. (1998) suggested the existence of different receptor subtypes for adrenomedullin in the human adrenal cortex.

Mazzocchi et al. (1999) found abundant [¹²⁵I]-adrenomedullin binding sites in both zona glomerulosa and adrenal medulla in the rat adrenal gland.

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A.1.1.29

Atrial Natriuretic Factor (ANF)

A.1.1.29.1

General Considerations

PURPOSE AND RATIONALE

The atria of mammalian hearts synthesize and secrete peptides with potent natriuretic and vasoactive properties known as ANF = atrial natriuretic factor (de Bold et al. 1981). The atrial natriuretic peptide hormonal system consists of a 126-amino acids prohormone synthesized within myocytes of the heart and stored in storage granules within the heart before release into circulation (Kangawa and Matsuo 1984; Oikawa et al. 1984; Vesely 1992). This hormonal system contains several peptides from the 126-amino acid-prohormone with blood pressure lowering, natriuretic, diuretic and/or kaliuretic properties (Martin et al. 1990; Vesely et al. 1994). Thus, peptides consisting of amino acid 1 to 30 (LANP = long-acting natriuretic peptide), 31 to 67 (vessel dilator), 79 to 98 (kaliuretic peptide) and 99 to 126 (ANF) each have blood pressure lowering, natriuretic, diuretic and/or kaliuretic properties both in humans and in animals. Human and rat atria predominantly secrete a peptide of 28 amino acid residues, ANF-(99–126), which represents the C-terminus of a precursor sequence of 126 amino acid residues. In addition, vessel dilator and LANP circulate as distinct entities after having been proteolytically cleaved from the rest of the amino terminus by proteases (Ackerman et al. 1997).

Plasma immunoreactive ANF-(99–126) concentration increases in normal rats after volume expansion, while infusion of the peptide lowers blood pressure in several animal models of hypertension.

An international standard for atrial natriuretic factor was established by an international collaborative study (Poole et al. 1988). Human ANF-(99–126) was synthesized, highly purified and distributed to several laboratories, who performed radioimmunoassays, ra-

dioreceptor assays and an *in vitro* assay using the vaso-relaxant activity in precontracted rat aortic strips.

The C-type natriuretic peptide is a 22-amino acid peptide that was initially identified in the central nervous system (Ogawa et al. 1992; Barr et al. 1996; Amin et al. 1996). The distribution of C-type natriuretic peptide, which has structural homology with atrial and brain natriuretic peptides and also similar activities, is wide and includes the endothelium, myocardium, gastrointestinal and genitourinary tracts. **Brain natriuretic peptide** has been described as a novel cardiac hormone (Nakao et al. 1991). Yasue et al. (1994) studied the localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. N-terminal pro-brain natriuretic peptide became a diagnostic screening tool to differentiate between patients with normal and left ventricular systolic function (Bay et al. 2003; Gardner et al. 2003).

Jiao and Baertschi (1993) reviewed the neural control of the endocrine rat heart. Stimulation of cardiac sympathetic nerves potently stimulates ANF secretion.

ANF inhibits proliferation in non-myocardial cells and is anti-hypertrophic in cardiomyocytes. Silberbach et al. (1999) reported that activation of an extracellular signal-regulated protein kinase is required for the anti-hypertrophic effect of atrial natriuretic factor in neonatal rat ventricular myocytes.

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A.1.1.29.2

Bioassay for ANF

PURPOSE AND RATIONALE

Matsui et al. (1987) described a rapid bioassay for quantification of atrial natriuretic polypeptides in rats with continuous recording of the conductivity of the urine, urine flow and blood pressure.

PROCEDURE

Male Sprague Dawley rats weighing 180–240 g are anesthetized with 60 mg/kg i.p. pentobarbital sodium. Anesthesia is maintained by injection of supplemental doses of pentobarbital sodium. After tracheotomy, catheters are placed into the left jugular vein and the right carotid artery for injection of samples or infusion of 10% mannitol in 0.9% saline and for blood pressure recording. Through a small suprapubic incision the bladder is cannulated for collection of urine, and the cannula is connected to a device for continuous measurement of urine conductivity, by which the electrolyte concentration is estimated. Urine flow rate

is recorded using a drop counter, and urine samples are collected in a plastic tube.

After completion of surgery, 0.6–0.8 ml of 10% mannitol in 0.9% saline is administered into the jugular vein and is continuously infused at a rate of 4.0 ml/h by a syringe pump. Following an equilibration of 45–60 min, the bioassay is started when the urine flow is increased to 50–75 μ l/min. All test samples with a volume of 100 μ l are directly injected into the jugular vein followed by a wash injection of \sim 30 μ l of saline. Mean arterial blood pressure, urine conductivity, and urine flow rate are simultaneously recorded.

For dose-response curves, serial dilutions of human ANF (α -hANP) and of test substance are prepared. Vehicle and various doses of α -hANP or test substance are injected in a randomized sequence. Immediately after the injection, urine is collected for 10 min. Urine volumes are determined by weighing, and urinary sodium and potassium concentrations are measured by flame photometry.

EVALUATION

Linear-regression analyses by the method of least squares are used for evaluating dose-response relationship. One-way analysis of variance for repeated measures and the Newman-Keuls test are used to detect statistical differences.

MODIFICATIONS OF THE METHOD

Petersen et al. (1988) determined atrial content and plasma levels of atrial natriuretic peptides in rats with chronic renal failure. The natriuretic activity in the bioassay was estimated as the increase in Na excretion in urine samples from the control period to the maximal natriuretic response.

Allen and Gellai (1987) measured cardioinhibitory effects of atrial peptide in conscious chronically instrumented rats. The hemodynamic and renal excretory responses were measured with and without replacement of urinary fluid losses.

Thibault et al. (1984) characterized the biological activities of atrial natriuretic factor-related peptides *in vivo* by a natriuretic bioassay and *in vitro* by relaxation of contracted intestinal smooth muscle (chick rectum).

Schiller et al. (1986) tested synthetic analogs of atrial natriuretic peptide in the rabbit aorta assay and in a bioassay monitoring suppression of aldosterone secretion from bovine zona glomerulosa cells.

Dlouha and McBroom (1986) measured diuretic and natriuretic activity of atrial extracts of taurine-treated normal and cardiomyopathic hamsters by urine flow and Na⁺ excretion in the rat bioassay.

St.-Louis and Schiffrin (1988) measured vasorelaxant effects of different atrial natriuretic peptides on rat aortic and mesenteric artery rings and compared the results with the potency of the same peptides to displace ^{125}I -labeled ANP on membrane preparations of aorta and of mesenteric vein bed.

Kohse et al. (1992) described a bioassay for quantitative determination of natriuretic peptides in human biological samples using bovine aortic and bovine kidney epithelial cultured cells. The amount of cyclic AMP produced by these cells was measured by radioimmunoassay.

Keckskemeti et al. (1996) studied the effects of atrial natriuretic peptide (ANP) on action potential characteristics in various (human, rabbit, guinea-pig) atrial and guinea pig ventricular papillary muscles. The data suggested that ANP inhibits the slow inward Ca^{2+} channel activity and facilitates the K^{+} channel activity.

Salt-sensitive hypertension was found in ANP knockout mice (Melo et al. 1998).

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A.1.1.29.3 Receptor Binding of ANF

PURPOSE AND RATIONALE

Schiffrin et al. (1985) described receptors for atrial natriuretic factor in the rat.

PROCEDURE

Synthetic ANF-(99–126) is iodinated with ^{125}I by a modification (Gutskowska et al. 1984) of the chloramine T method (Greenwood and Hunter 1963). Separation of radiolabeled ANF from free iodine is achieved by immunoaffinity chromatography followed by C-18 reverse phase high pressure liquid chromatography.

For preparation of membranes, Sprague Dawley rats weighing 300 g are sacrificed by decapitation. The atria, ventricles, renal arteries, mesentery, the mesentery vascular bed, and adrenals are processed for binding studies. Adrenal capsules are separated by manual compression. The tissues are immersed in 0.25 M sucrose solution, finely minced with scissors, and homogenized in a Polytron (setting 8, 10 s twice). The homogenate is centrifuged at 1550 g for 10 min at 4°C; the supernatant is decanted and recentrifuged. The final supernatant is filtered through a cheesecloth, then centrifuged at 10,400 g for 30 min. The pellet is resuspended in a 0.05 M Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM MgCl_2 , 0.5 mM phenyl methyl sulfonyl fluoride, 0.1% bacitracin, and 1 μM aprotinin. Proteins are measured by the Coomassie blue method (Spector 1978). Next, bovine serum is added at a concentration of 0.2%, and the membranes are diluted to a protein concentration of 0.25 to 1 mg/ml in the Tris-buffer containing 0.2% albumin.

The ^{125}I -ANF binding assay uses 30–50 pM of labeled ANF and 10^{-13} to 10^{-6} unlabeled ANF in competition experiments. In saturation experiments, increasing concentrations of ^{125}I -ANF (6–200 pM) are used, and nonspecific binding is determined by incubation in the presence of 1 μM unlabeled ANF for each point of the saturation curves. Incubation is done with 25–100 μg of receptor protein per tube, at 4°C for 60 min. All assays are performed in duplicate. Separation of bound and free radioactivity is achieved by rapid filtration through polyethylenimine-treated Whatman GF/C filters soaked with the assay buffer. The filters are washed twice with 3 ml of 0.9% NaCl,

then are allowed to dry and are counted in a gamma counter.

EVALUATION

Binding data are analyzed by computer assisted non-linear regression analysis using the LIGAND program (Munson and Rodbard 1980). The inhibition constant K_i is calculated according the Cheng and Prussoff equation.

MODIFICATIONS OF THE METHOD

Misono (2000) found that the binding of atrial natriuretic factor to its receptor is dependent on chloride concentration.

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A.1.1.29.4

ANF Gene Expression

PURPOSE AND RATIONALE

Production of atrial natriuretic factor and brain natriuretic peptide can be measured by gene expression using total RNA extraction and Northern blot analysis and the quantitative competitive reverse transcription polymerase chain reaction (Hama et al. 1995; Ogawa et al. 1996, 1997, 1998, 1999).

PROCEDURE

Extraction of Plasma and Tissue Samples

Plasma samples are acidified by adding 100 μl /ml of 1 mol/l HCl and passed through Sep-Pak C_{18} cartridges (Millipore) that are pre-wetted with 5 ml of 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) and 10 ml of 0.1% TFA. The cartridges with the absorbed peptides are washed with 20 ml of 0.1% TFA and eluted with 3 ml of 60% acetonitrile in 0.1% TFA.

Tissue samples are homogenized in 10 vol of an extracting mixture consisting of 0.1 N HCl, 1.0 mol/l acetic acid, and 1% NaCl and centrifuged at 10,000 g for 30 min at 4°C. The supernatants are then extracted with the use of Sep-Pak C_{18} cartridges by elution with 80% acetonitrile in 0.1% TFA. The eluates from tissue or plasma are freeze-dried and processed for RIA.

Total RNA Extraction and Northern Blot Analysis

Atrial and ventricular tissue samples from individual rats are extracted using Trizol (GIBCO BRL). Total RNA from the atrium (10 μg) and ventricle (20 μg) are electrophoretically separated in an agarose-formaldehyde gel followed by blotting to nylon membranes (Hybond N+, Amersham) overnight. Membranes are prehybridized in $2.5 \times$ Denhardt's solution, $5 \times$ SSC, 50% formamide, 25 mmol/l KH_2PO_4 , pH 6.4, 0.2% SDS, and 0.2 mg/ml herring sssDNA for 3 h at 42°C for cDNA probes, or prehybridized in $5 \times$ Denhardt's solution, $6 \times$ SSC, 50 mmol/l NaH_2PO_4 , 0.5% SDS, and 0.2 mg/ml herring sssDNA for 3 h at 5°C below the calculated T_m for oligonucleotide probes. Hybridization is then carried out for 16 h at the same temperature and the same solution as the prehybridization condition except for the presence of the radiolabeled probes. Five cDNA probes and two oligonucleotide probes are used. The cDNA probes used are as follows: (1) a 900-bp *EcoRI/HindIII* fragment containing the full-length rat ANF cDNA, (2) a 595-bp *SalI* fragment containing full-length rat BNP cDNA, (3) a 5-kb *EcoRI/SalI* fragment of the mouse 28S rRNA cDNA probe, (4) a 2-kb *BamHI/BglIII* fragment of the mouse PGK gene cDNA, and (5) rat α_1 -III collagen cDNA containing 1300 bp of the 3' noncoding and coding regions. The two oligonucleotide probes are 39 and 24 base fragments specific for unique regions in the 3' untranslated regions of the rat α -MHC and β -MHC genes. The α -sequence is 5'-GGGATAGCAACAGCGAGGCTCTTTCTGCTGGACAGGTTA-3' ($T_m = 60^\circ\text{C}$), and the β -sequence is 5'-CTCCAGGTCTCAGGGCTTCACAGG-3' ($T_m = 52^\circ\text{C}$).

The cDNAs are labeled with 5'-[α - ^{32}P]dCTP (3000 Ci/mmol, Amersham) using the Megaprime DNA labeling system (Amersham). The oligonucleotides are labeled with [γ - ^{32}P]ATP (3000 Ci/mmol, Amersham) using a 5'-end-labeling kit (Amersham). At the end of hybridization, the membranes are washed twice at 42°C with $2 \times$ SSC and 1% SDS and twice at 55°C with $1 \times$ SSC and 0.1% SDS for the cDNA probes or are washed once at 30°C with $5 \times$ SSC and 0.1% SDS and twice at the same temperature as the hybridization with $1 \times$ SSC and 0.1% SDS for

the oligonucleotide probes. Before additional probing, bound counts are completely stripped from the membranes by washing twice in 10 mmol/l sodium citrate, pH 6.8, 0.25% SDS for 10 min at 100°C. Autoradiographs are scanned with an Ultrascan XL laser densitometer (LKB Produkter) and LKB 2400 Gelscan XL software package. The scanning values of ANF, BNP, collagen-III, and α -MHC and β -MHC mRNAs are normalized to 28S ribosomal RNA or PGK mRNA as internal controls to correct for differences in the amount of RNA applied and transfer efficiency.

Plasma and cardiac tissue concentrations of immunoreactive ANF and BNP are determined by RIA with anti-rat ANF_{99–126} and anti-rat BNP_{64–95} sera, respectively, from Peninsula Laboratories.

Quantitative competitive reverse transcription polymerase chain reaction

RNA samples are reverse transcribed with Super Script II RNase H 2 Reverse Transcriptase and oligo(dT)_{12–18} primer with the use of a reverse transcription kit (GIBCO BRL). An aliquot of the cDNA product is used for PCR amplification with ANF primers. A dilution series of total RNA (5 mg) aliquots is prepared for each sample. Each dilution is spiked with competitor RNA. After the PCR, aliquots (5 ml) of the PCR product are electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Photographs are taken with Polaroid 55 film, and the negatives are scanned with the use of an Ultrascan XL laser densitometer and Gelscan XL 2000 software package. The ratio of the density of the competitor RNA to the target RNA is plotted against the amount of the competitor RNA added to each reaction.

EVALUATION

All results are expressed as mean \pm SEM. A level of $P < 0.5$ is considered significant. ANOVA is performed to determine statistical differences among multiple groups. When significance is obtained by ANOVA, Fisher's least squares difference post hoc analysis is used to determine pairwise differences.

MODIFICATIONS OF THE METHOD

Ramirez et al. (1997) reported that the nuclear δ_B isoform of Ca^{2+} /calmodulin-dependent protein kinase II regulates atrial natriuretic gene expression in cultured neonatal rat ventricular myocytes.

Thuerlauf et al. (1998) found that the p38 mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through

a serum response element and discussed the potential role for the transcription factor ATF6.

Kakita et al. (1999) studied p300 protein as a coactivator of the transcription factor GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene.

Bianciotti and de Bold (2000) investigated the effect of selective ET_A receptor blockade on natriuretic peptide gene expression in DOCA-salt hypertension in rats.

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Radioimmunoassay for ANF

PURPOSE AND RATIONALE

Gutkowska et al. (1984) developed a direct radioimmunoassay of atrial natriuretic factor (ANF). The

method uses a synthetic 26-amino-acid fragment (8–33 ANF) of the native peptide.

PROCEDURE

Because 8–33 ANF is a small molecule, it is necessary to covalently conjugate the peptide to a larger protein (bovine thyroglobulin) for immunization. To 50 mg thyroglobulin dissolved in 2 ml distilled water, pH 7.4, 30 mg CDI [1-ethyl-3-(3-dimethylamino-propyl) carbodiimide HCl] is added in 1 ml distilled water, pH 7.4. Then 5 mg 8–33 ANF in water is added dropwise while stirring. The solution is kept overnight at 4°C, then another 30 mg CDI is added and the mixture is kept for 2 h at room temperature with constant stirring. The cloudy mixture is dialyzed for 24 h at 4°C against 0.9% saline. The dialyzed material is then fractionated and stored at –70°C.

For immunization, 100 µg of the ANF-thyroglobulin complex are suspended in 1 ml saline, thoroughly mixed with 1 ml complete Freund's adjuvant and injected into the shaved backs of New Zealand white rabbits. Each animal receives also 0.5 ml *Bordetella pertussis* vaccine subcutaneously with the primary immunization. The animals are reimmunized at monthly intervals with 100 µg of antigen in incomplete Freund's adjuvant and bled by ear artery 10 days after the booster injection.

For iodination, 5 µg ANF in 5 µl 0.01 M ammonium acetate, pH 5.0 is introduced in a 1.5 ml Eppendorf vial followed by the addition of 1 mCi Na ¹²⁵I in a volume of 25 µl. Chloramine T 10 µg/10 µl is added to the reaction vial and 30 s later sodium metabisulfide (20 µg/10 µl) is added. Each addition is followed by mixing. Purification of the iodinated tracer is achieved by HPLC on a µBondapax C₁₈ column, eluted with a linear gradient of 20 to 50% acetonitrile with 0.1% trifluoroacetic acid with a slope of 0.5%/min and a flow rate of 1 ml/min.

The radioimmunoassay procedure is performed in polystyrene tubes at 4°C by mixing 100 µl of standard or sample, 100 µl of antiserum diluted 1:4000, 100 µl of ¹²⁵I-ANF and 300 µl of the same buffer containing 1% BSA. After incubation for 24 h at 4°C the free from antigen-bound ¹²⁵I-ANF is separated by dextran-coated charcoal. One ml of dextran-charcoal suspension is added to each tube. After 5 s agitation the tubes are centrifuged at 4000 rpm at 4°C for 10 min. The supernatant is decanted and the radioactivity counted in a gamma counter.

EVALUATION

Dose-response curves are prepared and Scatchard analysis is performed.

MODIFICATIONS OF THE METHOD

Radioimmunoassays were also developed for long-acting natriuretic peptide and vessel dilator (Vesely et al. 1994; Winters et al. 1989).

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A.1.1.30

Urotensin II

A.1.1.30.1

General Considerations

PURPOSE AND RATIONALE

Urotensin II is a cyclic peptide originally isolated from the urophysis, the hormone storage-secretion organ of the caudal neurosecretory system of teleost fishes, such as *Gillichthys mirabilis* (Pearson et al. 1980; Maguire and Davenport 2002).

Several structural forms of urotensin II have been reported in different species (Grieco et al. 2004). These peptides show smooth muscle contracting activity. Itoh et al. (1987) reported contraction of major artery segments of rat, especially of the thoracic aorta, by urotensin II.

Human urotensin II is an 11-amino-acid peptide that retains the cyclic portion typical of fish urotensin II. It has been found in vascular and cardiac tissues and is a very potent constrictor of certain human isolated arteries and veins as well as of several vessels of other species (Douglas et al. 2000; Douglas 2003).

The potency of urotensin II as a vasoconstrictor is an order of magnitude greater than that of endothelin-1, making human urotensin II the most potent vasoconstrictor identified so far.

Human urotensin II is also a potent endothelium-dependent relaxant in rat precontracted arteries (Katano

et al. 2000) and in human small pulmonary and systemic abdominal resistance arteries (Stirrat et al. 2001).

Bottrill et al. (2000) found that human urotensin II contracted endothelium-intact rat isolated left anterior descending coronary arteries. The contractile response was significantly enhanced by removal of the endothelium. However, human urotensin II caused concentration-dependent relaxation of 5-HT-precontracted arteries, which was abolished by *N*-nitro-L-arginine methyl ester (L-NAME) or removal of the endothelium.

Using merino ewes as experimental animals, Watson et al. (2003) found that urotensin II acts centrally to increase epinephrine and adrenocorticotrophic hormone (ACTH) release and causes potent inotropic and chronotropic actions.

Coy et al. (2002) investigated structural requirements at the N-terminus of urotensin II octapeptides.

Carotenuto et al. (2004) investigated the active conformation of urotensin II by CD spectroscopy and NMR analysis in SDS micelles.

Watson and May (2004) reviewed the role of urotensin II in central and peripheral cardiovascular control.

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Rat Thoracic Aorta Bioassay for Urotensin II

PURPOSE AND RATIONALE

Most studies on urotensin analogs, agonists and antagonists used the isolated thoracic aorta of rats as a pharmacological model (Itoh et al. 1988; Nothacker et al. 1999; Sauzeau et al. 2001; Camarda et al. 2002a; Grieco et al. 2002a, 2002b; Rossowski et al. 2002). Several urotensin II antagonists have been tested in this model (Behm et al. 2002; Herold et al. 2003).

Patacchine et al. (2003) tested urantide, an ultrapotent urotensin II antagonist peptide, in the rat aorta.

PROCEDURE

Male albino rats (Wistar strain, 275–350 g) are decapitated under ether anesthesia. The thoracic aorta is cleared of surrounding tissue and excised from the aortic arch to the diaphragm. From each vessel, a helically cut strip is prepared, and then it is cut into two parallel strips. The endothelium is removed by gently rubbing the vessel intimal surface with a cotton-tip applicator; the effectiveness of this maneuver is assessed by the loss of relaxation response to acetylcholine (1 μ M) in preparations precontracted with noradrenaline (1 μ M). All preparations are placed in 5-ml organ baths filled with oxygenated normal Krebs–Henseleit solution. Motor activity of the strips is recorded isotonicly (load 5 mN). A cumulative concentration–response curve to hU-II is constructed on one of the two strips, which serves as control. The other strip receives the antagonist peptide under examination and, after a 30-min incubation period, hU-II is administered cumulatively. Maximal contractile responses of preparations to hU-II are obtained by administration of KCl (80 mM) at the end of the cumulative curves.

EVALUATION

Antagonist activity is expressed in terms of pK_B (negative logarithm of the antagonist dissociation constant) and, assuming a slope of -1.0 , is estimated as the mean

of the individual values obtained with the equation: $pK_B = \log[\text{dose ratio} - 1] - \log[\text{antagonist concentration}]$ (Kenakin 1997). Competitive antagonism is checked by the Schild plot method: a plot with linear regression line and slope not significantly different from unity is considered as proof of simple reversible competition (Kenakin 1997).

MODIFICATIONS OF THE METHOD

Gibson et al. (1988) studied the influence of urotensin II on calcium flux in rat aorta. Urotensin II caused an increase in uptake of ^{45}Ca by segments of rat aorta. This increase was abolished by calcium channel blocking drugs.

Douglas et al. (2000) found differential vasoconstrictor activity of human urotensin II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey depending on species and anatomical localization.

Camarda et al. (2002b) studied the effects of human urotensin II in isolated vessels (aorta, large arteries, veins) of various species (rats, guinea pigs, rabbits, pigs, human) and compared them with other vasoactive agents (noradrenaline, angiotensin II, endothelin I).

Watanabe et al. (2001) found a synergistic effect of urotensin II with serotonin on rabbit vascular smooth muscle cell proliferation.

Tamura et al. (2003) examined the effects of urotensin II on activation of extracellular signal-regulated kinase and focal adhesion kinase in cultured vascular smooth muscle cells.

Tzanidis et al. (2003) studied direct actions of urotensin II on the heart in a rat model of heart failure after myocardial infarction and the implications for cardiac fibrosis and hypertrophy.

Matsushita et al. (2003) showed that urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line LLCPK1.

Behm et al. (2004) investigated the role of urotensin II in the etiology of essential hypertension. Intravenous injection in anesthetized cats induced an increase in systemic blood pressure and peripheral vascular resistance.

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Intracellular Calcium Mobilization Assay

PURPOSE AND RATIONALE

Human urotensin II induces concentration-dependent increases in intracellular calcium in HEK-293 cells expressing human GPR14 (Ames et al. 1999). Herold et al. (2003) used this assay to test a synthetic antagonist at human and rat urotensin II receptors.

PROCEDURE

Cell Culture

HEK293 cells stably expressing the hUT or rUT receptors are generated and propagated as described previously (Ames et al. 1999).

Intracellular Calcium (Ca^{2+}_i) Mobilization Assay

hUT-HEK293 cells or rUT-HEK293 cells are seeded in blackwalled, clear-bottomed 96-well Biocoat plates (Beckton-Dickinson, Bedford, Mass., USA, Herold et al. 2004) at a density of 45,000 cells/well, grown in the incubator at 37°C for 18–24 h, and prepared for Ca^{2+}_i measurements (Ames et al. 1999). Plates are placed into the Fluorometric Imaging Plate Reader (Molecular Devices, Sunnyvale, Calif., USA) where cells, loaded with Fluo 3 (Molecular Probes, Eugene, Ore., USA), are exposed to excitation (488 nm) from a 6-W argon laser. Fluorescence is monitored at 566 nm emission for all 96 wells simultaneously, and data are read every 1 s for 1 min and then every 3 s thereafter. Agonist is added after 10 s and concentration–response curves are obtained by calculating the maximal fluorescence counts above background after the addition of each concentration of agonist. For antagonist studies, BIM-23127 (Bachem, King of Prussia, Pa., USA) is added 10 min prior to the addition of hU-II (California Peptide Research, Napa, Calif., USA).

EVALUATION

Concentration–response curves are analyzed by nonlinear regression using GraphPad Prism 3.0 software (GraphPad, San Diego, Calif., USA).

MODIFICATIONS OF THE METHOD

Flohr et al. (2002) performed structure–activity relationship studies on urotensin II by investigating peptide analogs and their ability to mobilize calcium in GPR14-transfected CHO cells.

Camarda et al. (2002) evaluated a new ligand for the urotensin II receptor ($[Orn^8]U-II$) in calcium functional assays performed on HEK293 cells expressing the recombinant rat and human UT receptor.

Croston et al. (2002) used a functional mammalian cell-based R-SAT assay (ACADIA Pharmaceuticals, San Diego, Calif., USA) to identify non-peptide agonists of the CRP14/urotensin II receptor in high-throughput screening. According to Shapiro et al. (2002) NIH-3T3 cells were grown in 96-well tissue culture plates to 70%–80% confluence in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin/Gln. Cells were transfected for 12–16 h with plasmid DNAs using Superfect Reagent (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocols. R-SATs were performed with 0.5–50 ng/well receptor and 20 ng/well β -galactosidase plasmid DNA. After overnight transfection, medium was replaced with serum-free Dulbecco's modified Eagle's medium containing 2% cyto-sf3 (Kemp Biotechnologies, Frederick, Md., USA) and 1% penicillin/streptomycin/Gln and varying concentrations of drug. Cells were grown in a humidified atmosphere with 5% ambient CO_2 for 4–6 days. Medium was removed from the plates, and β -galactosidase activity was measured by the addition of *o*-nitrophenyl β -D-galactopyranoside (in phosphate-buffered saline with 5% Nonidet P-40 detergent). The resulting colorimetric reaction was measured using a spectrophotometric plate reader (Titertek, Huntsville, Ala., USA) at 420 nm. For HTS, NIH-3T3 cells transiently transfected with the urotensin II receptor expression vector and plasmid were frozen and for the assay thawed, plated and exposed to drug.

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Receptor Binding of Urotensin II

PURPOSE AND RATIONALE

The G-protein-coupled receptor GPR14/SENr was described by Marchese et al. (1995) and Tal et al. (1995). Liu et al. (1999) and Mori et al. (1999) identified urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. It was renamed as urotensin II (UT) receptor by UPHAR (Douglas and Ohlstein 2000). Human, rat, mouse and monkey receptors have been cloned. The amino acid sequence identity of the monkey UT receptor is 97% and 77% identical to the human and rat sequences respectively, while the mouse UT receptor is 76% and 93% identical to the human and rat sequences, respectively.

Brkovic et al. (2003) performed functional and binding characterizations of urotensin-II-related peptides in human and rat urotensin II receptor assays.

PROCEDURE

Reagents and Solvents

The following fluorenylmethyloxycarbonyl-protected amino acids were purchased from Chem-Impex International (Wood Dale, Ill., USA): Ala, Cys(Trt), His(Trt), Phe, Trp, Lys-(Boc), Tyr(tBu), Asp(OtBu), Glu(OtBu), Pro, Gln(Trt), Thr(tBu), Arg(Pbf), Orn(Boc), HomoCys(Trt), Cys(Acm), and D-Trp. Biograde TFA was obtained from Halocarbon (River Edge, N.J., USA). Diisopropylethylamine was from Aldrich (Milwaukee, Wis., USA). Wang resin and benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate were purchased from Albatross (Montreal, QC).

Basal ISCOVE medium and fetal calf serum (FCS) were from Biochrom (Berlin, Germany). The GC-melt PCR kit as well as the human and rat genomic DNA were purchased from CLONTECH (Palo Alto, Calif., USA). The pEAK8 mammalian episomal expression

vector and the selection marker puromycin were from Edge Biosystems (Gaithersburg, Md., USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, HEPES, LipofectAMINE reagent, and penicillin-streptomycin were from Invitrogen (Carlsbad, Calif., USA). The pCDNA3.1(+) mammalian expression vector was from Invitrogen. The calcium-sensitive fluorescence dye Fluo-4 and Pluronic F-127 were obtained from Molecular Probes (Eugene, Ore., USA). Flashplates PLUS and monoiodinated human [¹²⁵I-Tyr⁹]urotensin II for radioligand binding assays were from Perkin Elmer Life Sciences (Boston, Mass., USA). Gentamicin, the transfection reagent FuGene 6, and Complete protease inhibitor were purchased from Roche (Basel, Switzerland). Bacitracin, EDTA-disodium salt, probenecid, MgCl₂, NaCl, and sucrose were obtained from Sigma-Aldrich (St. Louis, Mo., USA).

Cloning of Human and Rat Urotensin II Receptor

As the putative human urotensin II receptor sequence is intronless, this protein was cloned from human genomic DNA via PCR. PCR conditions, established to amplify the human GPR14 sequence, were 94°C; 10 min followed by 35 cycles of 94°C, 1 min, 60°C, 1 min, 72°C, and 2 min using the GC-melt kit. Primers designed to amplify the coding sequence contained a *Bam*HI site in the forward and a *Xba*I site in the reverse primer, respectively. The urotensin receptor coding region, flanked by *Bam*HI/*Xba*I sites, was cloned into the pCDNA3.1(+) mammalian expression vector and sequenced in both directions. For generation of stable cell lines, the human U-II receptor coding sequence flanked by a 5' *Eco*RI site and a 3' *Eco*RV site was cloned into the mammalian episomal expression vector pEAK8.

The rat U-II receptor coding sequence flanked by a 5' *Eco*RI and 3' *Not*I site was amplified via PCR from rat kidney cDNA and cloned into the mammalian pEAK8 expression vector. Sequences of all urotensin-II-receptor-expressing plasmids were verified by dideoxy sequencing in both directions.

Cell Culture and Transfection

CHO K1-cells were grown in basal ISCOVE medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml), and 25 mg/ml gentamicin at 37°C in a humidified 5% CO₂ incubator. Cells were transiently transfected with the U-II receptor cDNAs using the LipofectAMINE reagent according to the manufacturer's protocol. After 18–24 h following the

transfection, cells were split into blackwalled 96-well plates at a density of 50,000 cells/well and cultured for an additional 18- to 24-h period before being used in the functional fluorescence imaging plate reader (FLIPR) assay (described below in detail) measuring intracellular Ca^{2+} release upon receptor activation.

FLIPR ASSAY

Cells were loaded in 96-well plates for 1 h (37°C , 5% CO_2) with 100 μl of PBS (without Ca^{2+} , Mg^{2+} and NaHCO_3) containing 4 μM of the fluorescent calcium indicator Fluo-4, 0.22% Pluronic F-127 in dimethyl sulfoxide, 2.5 mM probenecid, 1 mM EGTA, and 1% FCS. Cells were then washed three times with PBS (without Ca^{2+} , Mg^{2+} and NaHCO_3) containing 1 mM EDTA, 0.5 mM MgCl_2 , and 2.5 mM probenecid. After the final wash, a 100- μl residual volume remained on the cells. Peptides were aliquoted as $2\times$ solutions in 96-well plates and transferred by the instrument from the ligand plate to the cell plate. Fluorescence was recorded with the fluorometric imaging plate reader FLIPR (Molecular Devices, Sunnyvale, Calif., USA) over a period of 3 min. Fluorescence was recorded simultaneously in all wells at 3-s intervals during the first minute and at 10-s intervals during the last 2 min. Fluorescence data were generated in duplicate and repeated at least three times.

Generation of Stable Human and Rat U-II Receptor Expressing Cell Lines

For the generation of stable cell lines expressing the human and rat urotensin II receptor, HEK293 cells were transfected with human- and rat-pEAK8 constructs using the FuGene 6 transfection reagent according to the supplier's protocol. Two days after transfection, cells were selected in DMEM, supplemented with 10% FCS, 20 mM HEPES, penicillin-streptomycin (10,000 IU/ml – 10,000 $\mu\text{g}/\text{ml}$), and 1 $\mu\text{g}/\text{ml}$ puromycin for a period of 4 weeks (37°C , 5% CO_2 , 95% relative humidity). Functional activity of the urotensin-II-receptor-expressing cell population was verified with a FLIPR assay recording urotensin-II-mediated intracellular Ca^{2+} release, as described above.

Membrane Preparation and Radioligand Binding Assays

HEK293 cells stably expressing human or rat urotensin II receptors were cultured up to 80% confluency in DMEM, supplemented with 10% FCS, 20 mM HEPES, penicillin-streptomycin (10,000 IU/ml – 10,000 $\mu\text{g}/\text{ml}$), and 1 $\mu\text{g}/\text{ml}$ puromycin (37°C , 5%

CO_2 , 95% relative humidity). Cells were washed once with ice-cold PBS and a second time with PBS containing the protease inhibitor cocktail (Complete). Cells were scraped off and centrifuged gently. The pellet was resuspended in a buffer containing 5 mM HEPES, 1 mM EDTA-disodium salt, and the cocktail of protease inhibitor Complete and then incubated on ice for 15 min. Cells were pelleted again and resuspended with a homogenizer (Unit F8B, Constant cell disruption systems; Honiley, Warwickshire, UK). The supernatant and resuspended pellet were combined and centrifuged at 50,000 g (Beckman Avanti J251). The cell membrane pellet was resuspended in a buffer consisting of 20 mM HEPES, 1 mM EDTA-disodium salt, 150 mM NaCl, and 10% sucrose. Membrane aliquots were stored at -80°C . One day before the binding assay, membranes were thawed, pelleted, and resuspended in the assay buffer consisting of 20 mM HEPES, 150 mM NaCl, 1 mM EDTA-disodium salt, 160 $\mu\text{g}/\text{ml}$ bacitracin, and Complete protease inhibitor (two tablets per 100 ml).

Membranes were distributed into 96-well wheat-germ-agglutinin Flashplates PLUS, incubated overnight for adsorption, and then the Flashplates PLUS were washed twice with the assay buffer. For equilibrium binding assays, 0.2 nM human [^{125}I -Tyr9]urotensin II (initial specific activity 2200 Ci/mmol, or 81,400 GBq/mmol) was incubated with the indicated amounts of unlabeled competitors for 4 h and radioactivity counted in a 1450 Microbeta Wallac Jet (Wallac, Turku, Finland).

EVALUATION

Data of the FLIPR assay were analyzed by non-linear curve fitting using GraphPad Prism version 3.0 (GraphPad Software, San Diego, Calif., USA).

Non-specific binding was determined in the radioligand binding assays in the presence of 10 μM human U-II and corresponded to $31 \pm 2\%$ ($n=5$, mean \pm SEM) of the total binding for membranes expressing the human urotensin II receptor and to $8 \pm 1\%$ ($n=5$, mean \pm SEM) of the total binding for membranes expressing the rat urotensin II receptor. Data analysis was performed with GraphPad Prism 3.0.

MODIFICATIONS OF THE METHOD

Flohr et al. (2002) identified non-peptide urotensin II receptor antagonists by virtual screening on a pharmacophore model derived from structure–activity relationships and nuclear magnetic resonance studies on urotensin II.

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Urotensin II Gene Expression

PURPOSE AND RATIONALE

The UT receptor is expressed in vascular tissue (Ames et al. 1999; Maguire et al. 2000). A direct link between U-II, the UT receptor and vasoconstriction has to be assumed (Ames et al. 1999; Douglas and Ohlstein 2001). Behm et al. (2003) used homologous recombination in embryonic stem (ES) cells to generate mice lacking the UT receptor coding region to determine the effect(s) of this receptor on vascular reactivity both *in vivo* and *in vitro* and to verify that U-II exerts its effects on vascular smooth muscle tone via an interaction with UT.

PROCEDURE

Targeting the UT Gene and Generation of Mutant Mice

Gene targeting was performed in murine E14.1 ES cells, replacing the single coding exon of the UT receptor locus with a positive selection cassette containing the neomycin phosphotransferase gene (Neo) driven by the phosphoglycerate kinase I (PGK) promoter. 5'- and 3'-homology arms, both of ~4.0 kb, were cloned from a 129SVJ mouse genomic bacterial artificial chromosome (BAC) library and placed on either side of the positive selection cassette. Homologous recombination in neomycin-resistant ES cells was confirmed by Southern blot of *Bam*HI-digested genomic DNA using an ~800-bp *Bam*HI/*Sma*I restriction fragment as the 5' external probe (which detects 6.5- and 6.0-kb bands at the wild-type and targeted locus, respectively). Approximately 1 in 80 G418-resistant clones had undergone homologous recombination. Homologous recombination at the 3' end was confirmed in these ES cell clones by Southern blot of *Hind*III-digested genomic DNA using a ~700-bp *Xmn*I/*Hind*III restriction fragment as the 3'-external probe (which detects 5.5- and 5.0-kb bands at the wild-type and targeted locus, respectively). Three targeted clones were injected into C57B16/J-derived blastocysts. Male chimeras were crossed with C57B16/J females to give N1F0 offspring, which were subsequently intercrossed to generate N1F1 offspring. In addition, N1F0 offspring were successively backcrossed to C57B16/J females to generate N5F0 mice. These were intercrossed to create an N5F1 population.

Genotyping of Study Populations

N1F1 and N5F1 study populations were genotyped by polymerase chain reaction (PCR) and Southern blot of genomic DNA isolated from the hearts of animals used in the studies. Hearts of wild-type (UT^(+/+)) and UT receptor knockout (UT^(-/-)) mice were cut into small pieces (~1 mm) and placed into polypropylene tubes. Extraction buffer [2 ml; 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 0.5% SDS, 20 µg ml⁻¹ RNase, 100 µg ml⁻¹ proteinase K] was added and samples were incubated at 50°C–55°C for 4 h until completely lysed. The mixture was then extracted two times each with phenol, phenol/chloroform and chloroform (Maniatis et al. 1989). Genomic DNA was precipitated by adding 2.5 vols of cold ethanol, washed with 70% v/v ethanol (–20°C) and dissolved in 200 µl of 10 mM Tris-HCl, 1 mM EDTA TE, pH 8.0. Once dissolved, the purity and concentration of the DNA

were measured by spectrophotometry (absorbency at 260 and 280 nm wavelength).

PCR amplification was performed using 50- μ l aliquots (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 17 mM MgCl₂, 200 nM 2'-deoxynucleotide 5'-triphosphates (dNTPs), 10% v/v dimethylsulfoxide, 1.25U *Taq* DNA polymerase; (Perkin-Elmer, Norwalk, Conn., USA) using 200 ng of genomic DNA as the template and PCR primers specific to the neomycin resistance gene present at the targeted locus (5'-TGA ACA AGA TGG ATT GCA CGC AGG TTC TCC GGC-3' and 5'-GCC AAG CTC TTC AGC AAT ATC ACG GGT AGC-3', yielding an ~700-bp product)) and mouse UT gene-specific primers (5'-CTG GCT GAC CTG CTG TAT CTG CT-3' and 5'-CAG GGT CAC ACA AAG CAC TCT CA-3', yielding an ~900-bp product). A 500-bp mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon was used as the internal control (5'-TGG CCA AGG TCA TCC ATG AC-3' and 5'-GTC CAC CAC CCT GTT GCT GTA G-3', yielding an ~500-bp product). Amplification was performed for 30 cycles at 60°C annealing for 30 s, 72°C extension for 90 s and 94°C denaturing for 30 s. Amplification of a 500-bp (GAPDH)/700-bp doublet alone corresponded to a UT^(-/-) genotype and a 500-bp (GAPDH)/900-bp doublet alone corresponded to a UT^(+/+) genotype.

Genomic DNA (20 μ g) was digested with *Bam*HI and run on an agarose gel (1%). The agarose gel was then treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, followed by neutralization with 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30–40 min. The DNA was transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, Mass., USA) and probed with cDNA corresponding to the full-length mouse UT receptor open reading frame (ORF). cDNA fragments were labeled with [α -³²P] 2'-deoxycytidine 5'-triphosphate (dCTP) using standard random primed methods (T7 Quick-Prime; Pharmacia Biotech, Piscataway, N.J., USA). Membranes were prehybridized for 2 h at 42°C and incubated overnight at 42°C with 1×10^9 cpm μ g⁻¹ denatured radiolabeled probe in standard buffer [50% deionized formamide, 6 \times sodium chloride, sodium citrate (SSC), 5 \times Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 μ g ml⁻¹ denatured, fragmented salmon sperm DNA]. Membranes were washed under conditions of low stringency (three 15-min washes in $1 \times$ SSC, 0.1% SDS at 28°C) followed by a high stringency wash in $0.1 \times$ SSC/0.1% SDS for 30 min at 55°C. Hybridization signals were detected by conventional X-ray autoradiography (Hyper film, Amersham

Life Science, UK) and phosphor imaging (Storm 860, Molecular Dynamics, Sunnyvale, Calif., USA).

Hemodynamics and Echocardiography

Male wild-type (UT^(+/+)) and homozygous UT receptor knockout (UT^(-/-)) mice, anesthetized with 1.5% isoflurane, underwent transthoracic echocardiographic determination of left ventricular end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), cardiac output (CO) and ejection fraction (EF). Further to this, mice were re-anesthetized the following day for hemodynamic evaluation where a fluid-filled catheter was inserted into the left carotid artery for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The catheter was then advanced into the left ventricle (LV) to obtain measurements of left ventricular end-systolic (LVESP) and end-diastolic pressure (LVEDP). At the end of the study, selected organs (right and left kidney, heart, right and left ventricle and lungs) were isolated and wet weights were measured.

Preparation and Utilization of Mouse Isolated Aortae and Mesenteric Arteries

Male (4 months; 27 g) wild-type (UT^(+/+)) and UT receptor knockout (UT^(-/-)) mice were anesthetized with inhaled isoflurane (5% in O₂) and killed by cervical dislocation. Proximal descending thoracic aortae were isolated and cleaned of adherent tissue. Vessels approximately 3 mm in length were suspended in 10-ml organ baths containing Krebs solution of the following composition (mM): NaCl 112.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25.0, dextrose 11.0. Krebs solution was maintained at $37 \pm 1^\circ\text{C}$ and aerated with 95% O₂, 5% CO₂ (pH 7.4). For contraction studies, vessels were denuded of endothelium by rubbing with a fine forceps and indomethacin (10 mM) was added to the buffer. Changes in isometric force were measured under 0.5 g optimal resting tension using FT03 force-displacement transducers (Grass Instruments, Quincy, Mass., USA) coupled to Model 7D polygraphs.

The Halpern–Mulvany wire myograph (Model 610M; Danish Myo Technology, Denmark) was used for measurement of isometric force development of endothelium-intact superior mesenteric arteries (optimal resting tension of 0.5 g) and data were recorded using a Grass 7400 direct thermal recorder.

Following a 60-min equilibration period, vessels were treated with standard concentrations of KCl (60 mM) and phenylephrine (1 mM) to which subsequent agonist-induced responses were normalized.

Once the contractile response to phenylephrine had plateaued, carbachol (10 μ M) was added to the vessels in order to evaluate endothelial integrity.

Cumulative concentration–response curves to phenylephrine (0.1 nM to 10 μ M), angiotensin II (0.1 nM to 10 μ M), endothelin-1 (0.1 nM to 1 mM) and hU-II (0.01 nM to 3 μ M) were obtained for each vessel by adding the spasmogen to the tissue bath in half-log increments. During relaxation studies, vessels were precontracted with an EC₈₀ concentration of phenylephrine and contractile tone was reversed by adding cumulative amounts of carbachol (1 nM to 30 μ M) or sodium nitroprusside (0.1 nM to 1 μ M). Each response was allowed to plateau before the addition of subsequent agonist concentrations. Vessels were allowed to recover for at least 30 min between subsequent agonist–response curves, and were not exposed to subsequent agonists after treatment with either endothelin-1 or hU-II.

EVALUATION

All values are expressed as mean \pm SEM and *n* represents the total number of animals from which the vessels were isolated. Statistical comparisons were made using an unpaired, two-tailed *t*-test or Fisher's exact tests and differences were considered significant when $P < 0.05$. Concentration–response curves were fitted to a logistic equation as previously described (Douglas et al. 1995).

MODIFICATIONS OF THE METHOD

Coulouarn et al. (1998) reported cloning of the cDNA encoding the urotensin II precursor (Prepro-UII cDNA) in frog and human. Intense expression of the urotensin gene in motoneurons of the spinal cord was found indicating important physiological functions of urotensin II.

Elshourbagy et al. (2002) reported molecular and pharmacological characterization of genes encoding urotensin II peptides and their cognate G-protein-coupled receptors from the mouse and monkey. Monkey and mouse preproU-II genes were identified to encode 123 and 125 amino acids. Monkey and mouse UT receptors were 389, and 386 amino acids, respectively. Expression of mouse and monkey U-II/UT receptor mRNA was found also in extravascular tissues, including lung, pancreas, skeletal muscle, kidney and liver.

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Apelin

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General Considerations

PURPOSE AND RATIONALE

The peptide apelin, consisting of 36 amino acids, is found increasingly to play a role in biology and medicine (Kleinz and Davenport 2005; Masri et al. 2005). The family of apelin peptides is derived from a single gene and activates the 7-transmembrane G-protein-coupled receptor APJ. In the search for an endogenous ligand of the orphan G-protein-coupled receptor APJ, Tatamoto et al. (1998) isolated and characterized endogenous peptide ligands, designated apelin, from bovine stomach extracts. Apelin-13, consisting of 13 amino acids, was more active than apelin-36. APJ was first identified in a human gene by O'Dowd et al. (1993), sharing close identity to the angiotensin receptor. In rats, the greatest expression of APJ mRNA was detected in the lung, suggesting that APJ and its ligand play an important role in the pulmonary system

(Hosoya et al. 2000). Cloning, pharmacological characterization and brain distribution of the rat apelin receptor were reported by De Mota et al. (2000), and the physiological role of apelin and its receptor in rat brain by Reaux et al. (2001). The hypothalamic and hypophyseal distribution of the receptor suggested an involvement of apelin in the control of neuro- and adeno-hypophyseal hormone release, whereas the presence in the pineal gland and in discrete higher brain structures pointed to possible roles in the regulation of circadian rhythms and of water and food intake behavior. Studying the distribution of apelin-synthesizing neurons in the adult rat brain, Reaux et al. (2002) suggested multiple roles of apelin especially in the central control of ingestive behaviors, pituitary hormone release and circadian rhythms. Studying apelin immunoreactivity in the rat hypothalamus and pituitary, Brailoiu et al. (2002) concluded that apelin may be a signaling peptide released from the hypothalamic-hypophyseal axis. De Mota et al. (2004) found that apelin is a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. Masri et al. (2002) reported that apelin activates extracellular signal-regulated kinases via a system sensitive to pertussis toxin (PTX). Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin were reviewed by Medhurst et al. (2003). Jászberényi et al. (2004) studied the behavioral, neuroendocrine, and thermoregulatory actions of apelin-13. Wang et al. (2004) investigated the localization of apelin in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. Boucher et al. (2005) found in isolated mouse and human adipocytes that apelin is upregulated by insulin and obesity.

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Cardiovascular Actions of Apelin

PURPOSE AND RATIONALE

Most reports concentrate on the functional role of apelin in the cardiovascular system (Tatemoto et al. 2001; Katugampola et al. 2002; Sokodi et al. 2002; Katugampola and Davenport 2003; Chen et al. 2003; Berry et al. 2004; Kleinz and Davenport 2004; Kagiya et al. 2005; Losano 2005).

Cheng et al. (2003) studied the venous dilator effect of apelin in conscious rats using a method for measurement of body venous tone published by Pang (2000).

Ashley et al. (2005) found that apelin improves cardiac contractility and reduces cardiac loading *in vivo* in mice.

PROCEDURE

Male C57Bl/6 mice were used. The following studies were performed: magnetic resonance imaging of the heart, pressure–volume hemodynamics, effects of chronic apelin infusion, assessment of ventricular hypertrophy after sacrifice, and expression of the APJ receptor.

Pressure–volume hemodynamics were assessed using the Aria system (Millar Instruments, Houston, Tex., USA). This measurement platform, specifically designed for small rodents, comprises an ultra-miniature 1.4F (0.47 mm outer diameter) catheter, which incorporates pressure and conductance sensors, possessing hardware including analog-digital conversion and analysis software. Pressure is measured directly in mmHg, while the conductivity of blood is used to estimate volume and allow construction of pressure–volume relationships in real time.

Male mice aged 8–16 weeks were anesthetized with 1%–2% isoflurane in oxygen. The internal jugular vein was cannulated with PE tubing and a 10% albumin solution infused at 5 μ l/min following a bolus of 150 μ l over 5 min. After tracheostomy, a 19-gauge cannula was inserted into the trachea and the animal was ventilated at a tidal volume of 200 μ l at 100 breaths per minute. Following an incision just dorsal to the xyphoid cartilage, the diaphragm was visualized from below, and after diaphragmatic incision, the left ventricular apex was visualized. The pressure–volume catheter was inserted along the long axis of the left ventricle, from where it was adjusted to obtain rectangular-shaped pressure–volume loops. Baseline loops were recorded following volume replacement, at which point the inferior vena cava was visualized within the chest and occlusion parameters were recorded during and after a 5-s manual occlusion of the vessel. Next, the albumin solution was replaced by one containing 100 nM apelin, which was infused at 5 μ l/min for 20 min, following which baseline and occlusion loops were recorded once again.

Signals from the catheter were digitized using the Powerlab system and stored for offline analysis using the PVAN software. This allows analyses of pressure (e.g., end-systolic pressure, end-diastolic pressure) and derivation of pressure–time and volume–time parameters at steady state.

For **magnetic resonance imaging**, male C57Bl/6 mice aged 16 weeks were scanned twice on subsequent days. The animals underwent general anesthesia while breathing spontaneously via a nose cone fitted carefully to minimize escape of anesthetic into the environment. Two percent isoflurane was adminis-

tered with an oxygen flow rate of 1–2 l/min. Platinum needle ECG leads were inserted subcutaneously. Respiration was monitored by means of a pneumatic pillow sensor positioned against the abdomen. Mouse body temperature was maintained during scanning at 37°C by a flow of heated air thermostatically controlled by a rectal temperature probe. Magnetic resonance images were acquired on a 4.7 T Oxford magnet controlled by a Varian Inova console (Varian, Palo Alto, Calif., USA) using a transmit–receive, quadrature, volume coil with an inner diameter of 3.5 cm. Image acquisition was gated to respiration and to the ECG R wave (SA Instruments, Stony Brook, N.Y., USA). Coronal and sagittal scout images led to the acquisition of multiple contiguous 1-mm-thick, short axis slices orthogonal to the interventricular septum. Nine cine frames were taken at each slice level with the following sequence parameters: TE = 2.8 ms, NEX = 12, FOV = 3 \times 3 cm, matrix = 128 \times 128, flip angle = 60°. Cine frames were spaced 16 ms apart and acquired through slightly more than one cardiac cycle guaranteeing acquisition of systole and diastole. On the second day of scanning, mice received 300 μ g/kg body weight of apelin as an intraperitoneal injection 1 h prior to scanning. A pilot study had previously identified 1 h as an appropriate time within which to identify apelin effects resulting from peritoneal absorption. Planimetry measurements of end-diastolic and end-systolic dimensions were derived offline from short axis views of the left ventricle at the level of the papillary muscles using ImageJ software (National Institutes of Health, Bethesda, Md., USA). Ejection fraction was calculated as $[LVEDA-LVESA]/LVEDA$, where LVEDA is left ventricular end-diastolic area and LVESA is left ventricular end-systolic area.

EVALUATION

Data were analyzed using Student's *t* statistic (paired) or repeated measures analysis of variance using the post hoc comparison of Fisher.

MODIFICATIONS OF THE METHOD

Katugampola et al. (2001, 2002) described [¹²⁵I](Pyr¹)apelin as a radioligand for localizing the APJ orphan receptor in human and rat tissues.

Kasai et al. (2004) found that apelin worked as an angiogenic factor in the retinal endothelial cell line RF/6A.

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A.1.1.32

TRP Channels

A.1.1.32.1

General Considerations

The transient receptor potential (TRP¹) ion channels are named after the role of the channels in *Drosophila* phototransduction (Montell 2001). The mammalian genes are encoded by at least 28 channel subunit genes (Clapham 2003; Moran et al. 2004; Clapham et al. 2005). Six protein families comprise the mammalian TRP superfamily: the classic TRPs (TRPCs), the vanilloid receptor TRPs (TRPVs), the melastatin or long TRPs (TRPMs), the mucolipins (TRPMLs), the polycystins (TRPPs), and ankyrin transmembrane protein 1 (ANKTM1, TRPA1). The TRP channel primary structures predict six transmembrane (TM) domains with a pore domain between the fifth (S5) and sixth (S6) segments and both C and N termini presumably located intracellularly (Vannier et al. 1998).

The TRP superfamily includes >20 related cation channels that play critical roles in processes ranging from sensory physiology to vasorelaxation and male fertility. Defects in TRP channels have been associated with changes in growth control and one TRP-related protein may be a tumor suppressor. Moreover, mutations in a member of the TRP superfamily are a common cause of polycystic kidney disease, while disruption of another is responsible for mucopolidosis, a neurodegenerative disease. TRP proteins are widely expressed in the nervous system, and, in non-excitabile cells, TRP-related channels may be the primary mode of Ca²⁺ entry. TRP proteins are cation channels; however, they vary significantly in their selectivity and mode of activation. Nevertheless, members of the TRP superfamily share significant sequence homology and predicted structural similarities.

Based on primary amino acid homology, the conventional TRP proteins can be classified into three subfamilies: TRPC, TRPV, and TRPM (Birbaumer et al. 2003). Three additional, more distantly related subfamilies have recently been defined: ANKTM1, a Ca²⁺-permeant, non-selective cation channel, is the only mammalian member of the TRPA branch of TRP proteins. It is activated by noxious cold temperature, pungent mustard oil compounds and may additionally serve as a mechanosensitive transduction channel in auditory hair cells as well as an ionotropic cannabinoid receptor. The three mucolipins, TRPML1, 2, and 3, appear to be ion channels in intracellular vesicles. Mutations in TRPML1 cause mucopolidosis type IV,

a neurodegenerative lysosomal storage disorder, while genetic defects in TRPP2, a member of the TRPP subfamily, are frequently encountered in patients suffering from autosomal dominant polycystic kidney disease.

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- 1997; Lintschinger et al. 2000; Strübing et al. 2003), TRPC1 might be a component of different heteromeric TRP complexes. The subgroup most closely related to TRPC1 comprises TRPC4 and TRPC5. TRPC4 and TRPC5 are PDZ motif-containing proteins that can form homomeric cation channels that are activated following stimulation of G_q -coupled receptors (Okada et al. 1999) as well as receptor tyrosine kinases (Schaefer et al. 2000).
- Xu and Beech (2001) found that TRPC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells.
- Vandebrouck et al. (2002) described involvement of TRPC in the abnormal calcium influx observed in dystrophic (*mdx*) mouse skeletal muscle fibers.
- Venkatachalam et al. (2003) discussed regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C.
- Bergdahl et al. (2003) found that cholesterol depletion impairs vascular reactivity to endothelin-1 by reducing store-operated Ca^{2+} entry dependent on TRPC1.
- Amiri et al. (2003) published FRET-based analysis of TRPC subunit stoichiometry.
- Jho et al. (2005) reported that angiotensin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca^{2+} influx.
- Rao and Kaminski (2006) found that induction of intracellular elevation by Δ^9 -tetrahydrocannabinol in T cells involves TRPC1 channels.
- Takai et al. (2004) distinguished two types of non-selective cation channels by muscarinic stimulation with carbachol in bovine muscle cells.
- Kumar et al. (2006) studied upregulated TRPC1 channel in vascular injury *in vivo* and its role in human neointimal hyperplasia.
- Less information is available about **TRPC2**, which shares approximately 30% sequence identity with the TRPC3/6/7 subfamily. Full-length TRPC2 mRNA and several N-terminal splice variants have been found in mouse and rat tissue, but TRPC2 seems to be a pseudogene in humans (Vannier et al. 1999; Liman 2003). TRPC2 protein was localized to neuronal microvilli in rat vomeronasal organ (Liman 2003) and in the head of mouse sperm (Jungnickel et al. 2001).
- TRPC3**, **TRPC6**, and **TRPC7** are 75% identical. When expressed they constitute non-selective cation currents that rectify in both the inward (- voltages) and outward (+ voltages) directions. TRPC3, TRPC6, and TRPC7 are inwardly and outwardly rectifying, have relatively low selectivity for Ca^{2+} over Na^+ , and are ac-

A.1.1.32.2

TRPC Channels

The TRPC family can be divided into three subgroups by sequence homology as well as functional similarities: C1/C4/C5, C3/C6/C7, and C2. **TRPC1** was the first member of the mammalian TRP family purported to form an ion channel (Zitt et al. 1996). Given the widespread expression of TRPC1 and its ability to coassemble with other TRPC subunits (Xu et al.

tivated by diacylglycerol (DAG) (Hofmann et al. 1999; Okada et al. 1999; Putney et al. 2004). These channels seem to play important roles in vascular and airway smooth muscle (Trebak et al. 2003; Yu et al. 2003; Corteling et al. 2004).

Groschner and Rosker C (2005) described TRPC3 as a versatile transducer molecule that serves integration and diversification of cellular signals.

Graziani et al. (2006) investigated the potential role of membrane cholesterol as a regulator of cellular TRPC3 conductance.

Smyth et al. (2006) found a dissociation of regulated trafficking of TRPC3 channels in the plasma membrane from their activation by phospholipase C.

TRPC4 and TRPD5 are receptor-operated Ca^{2+} -permeable non-selective cation channels (Plant and Schaefer 2003).

Odell et al. (2005) found that epidermal growth factor induces tyrosine phosphorylation, membrane insertion and activation of transient receptor potential channel 4.

Saleh et al. (2006) described that angiotensin II activates two cation channels with distinct TRPC1 and TRPC6 channel properties in rabbit mesenteric artery myocytes.

Shimizu et al. (2006) reported that Ca^{2+} -calmodulin-dependent myosin light chain kinase is essential for activation of TRPC5 channels expressed in HEK293 cells.

Shi et al. (2004) described multiple regulation by calcium of murine homologs of transient receptor potential proteins TRP6 and TRP7 expressed in HEK293 cells.

Xu et al. (2006) identified sphingosine-1-phosphate as an activator of the calcium channel TRPC5, which controls vascular smooth muscle activity.

Estacion et al. (2004) studied activation of human TRPC6 channels by receptor stimulation.

Basora et al. (2003) reported that 20-hydroxyeicosatetraenoic acid (20-HETE) activates mouse TRPC6 channels expressed in HEK293 cells.

Maruyama et al. (2006) found that heteromultimeric TRPC6-TRPC7 channels contribute to arginine vasopressin-induced cation current of A7r5 vascular smooth muscle cells.

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A.1.1.32.3 TRPM Channels

The TRPM subfamily has eight members divided into four groups: M1 (melastatin)/M3, M7 (TRP-PLIK)/M6, M2/M8, and M4/M5 (Hartzeneck 2005; Fonfria et al. 2006). **TRPM1** may be regulated through direct interaction with a cytosolic isoform generated by alternative RNA splicing (Xu et al. 2001). **TRPM2** is a 1503-amino-acid protein that is highly expressed in brain (Nagamine et al. 1998) and present in blood cells.

Perraud et al. (2003) reviewed TRPM2 Ca^{2+} -permeable cation channels.

Hill et al. (2004a, 2004b) reported inhibition of TRPM2 channels by the antifungal agents clotrimazole and econazole as well as by flufenamic acid; Kraft et al. (2006) by *N*-(*p*-amylcinnamoyl)anthranilic acid.

Identified first by sequencing projects, the function of **TRPM3** is poorly understood. The hTRPM3 gene maps to human chromosome 9q-21.12 and encodes a 1555-amino-acid protein.

TRPM4 and **TRPM5** have similar characteristics. TRPM4b, a splice variant of TRPM4, and TRPM5 are Ca^{2+} -activated, voltage-modulated, monovalent-selective cation channels with 25-pS single-channel conductances (Launay et al. 2002; Hofmann et al. 2003; Nilius et al. 2003). Pérez et al. (2003) studied the ion channel TRPM5 in taste receptor cells.

TRPM6 and **TRPM7** comprise a unique subfamily of TRP proteins with both channel and kinase activities. TRPM7, which has 1863 amino acid residues, was identified in a yeast two-hybrid screen as a protein interacting with phospholipase C (PLC) (Runnels et al. 2001; Schmitz et al. 2003). It seems to be ubiquitously expressed. Takezawa et al. (2004) described receptor-mediated regulation of the TRPM7 channel through its endogenous protein kinase domain.

TRPM8 is a 1104-amino-acid protein that does not seem to contain associated enzymatic domains. TRPM8 is a non-selective, voltage-modulated conductance. At colder temperatures (8°C–28°C) or in the presence of menthol, TRPM8 current is activated at a more physiological range of voltages (Brauchi et al. 2004; Voets et al. 2004). This channel is expressed in

small-diameter primary sensory neurons, where it presumably functions as a thermosensor (McKemy et al. 2002; Peier et al. 2002).

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A.1.1.32.4 TRPV Channels

The TRPV subfamily is named after the first mammalian member of the subfamily, vanilloid receptor 1 (Caterina et al. 1997; Benham et al. 2002; Montell et al. 2002; Clapham 2003; Mutai and Heller 2003). TRPV5 and TRPV6 are highly Ca²⁺-selective TRP channels that mediate trans-epithelial Ca²⁺ transport in kidney and intestine (Hoenderop et al. 2003).

The TRPV channel subfamily has six members divided into two groups: V1/V2/V3/V4 and V5/V6. The vanilloid receptor, TRPV1, is the best understood ion channel in this class (Caterina et al. 1997; Caterina and Julius 2001).

TRPV channels as temperature sensors were reviewed by Benham et al. (2003).

The expressed **TRPV1** capsaicin receptor is a heat/proton/lipid/voltage-modulated Ca²⁺-permeant (P_{Ca}/P_{Na} 10) ion channel. A more voltage-gating-centric explanation is that at warmer temperatures (> 37°C) or in the presence of capsaicin, TRPV1 current is activated by a more physiological range of voltages (Brauchi et al. 2004). TRPV1 is desensitized by internal Ca²⁺; it is not activated by store depletion. TRPV1, V2, and V3 are activated by the synthetic compound 2-aminoethoxydiphenylborate (2-APB) (Chung et al. 2004; Hu et al. 2004). Endogenous cannabinoid receptor ligands, such as anandamide, are potential TRPV1 agonists.

The vanilloid receptor-like channel **TRPV2** is 50% identical to TRPV1, but is insensitive to capsaicin (Caterina et al. 1999). Like TRPV1 it is more permeable to Ca²⁺ than to Na⁺ (P_{Ca}/P_{Na} = 3:1). It has been proposed to mediate high-threshold noxious heat sensation, perhaps in the lightly myelinated A nociceptors, but its presence in non-sensory tissue suggests other functions as well.

TRPV3 is expressed widely but most strikingly in skin. Increasing temperature from 22 to 40°C in mammalian cells transfected with hTRPV3 elevates intracellular calcium by activating a non-selective cationic conductance (P_{Ca}/P_{Na} 10:1) (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002). As in sensory neurons, the current is steeply dependent on temperature, sen-

sitizes with repeated heating, and displays a striking hysteresis on heating and cooling, but the extent of expression in sensory neurons is controversial. Based on these properties, TRPV3 is thermosensitive in the physiological range of temperatures between TRPM8 and TRPV1 and may play a role in pain.

TRPV4 is 40% identical to TRPV1 and TRPV2 (Liedtke et al. 2000; Strotmann et al. 2000). When expressed in mammalian cells it comprises a moderately selective cation channel ($P_{Ca}/P_{Na}=6$), which, like TRPV1, displays a gently outwardly rectifying *I-V* relation. TRPV4 is 40% identical to TRPV1 and TRPV2 (Liedtke et al. 2000; Strotmann et al. 2000). When expressed in mammalian cells it comprises a moderately selective cation channel ($P_{Ca}/P_{Na}=6$), which, like TRPV1, displays a gently outwardly rectifying *I-V* relation.

TRPV5 and TRPV6 comprise a separate subfamily of TRPVs with only 30% identity with TRPV1. The expressed channels strongly inwardly rectify and are the most Ca^{2+} -selective ($P_{Ca}/P_{Na} > 100$) (Nilius et al. 2000; Vennekens et al. 2000; Yue et al. 2001; Den Dekker et al. 2003) of all TRP channels. These properties are consistent with proposed mechanisms for Ca^{2+} -selective channels in which negatively charged glutamic or aspartic acid residues provide a binding site for divalents within the pore. Intra- and extracellular $[Ca^{2+}]$ (Yue et al. 2001; Hirnet et al. 2003; Bödding and Flockerzi 2004) and calmodulin (Lambers et al. 2004) regulate TRPV6 activity. The localization of TRPV5 and TRPV6 to the proximal small intestine and collecting duct of the kidney, along with mouse knockout data, suggests that this family is important in calcium uptake via epithelial cells (Hoenderop et al. 2005).

Lee et al. (2005) reported that PIP_2 activates TRPV5 and releases its inhibition by intracellular Mg^{2+} .

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A.1.1.32.5

TRPV1 Receptor Assay

PURPOSE AND RATIONALE

The vanilloid receptor-1 (TRPV1) is a non-selective cation channel, predominantly expressed by peripherally sensory neurons, which is known to play a key role in the detection of noxious painful stimuli, such as capsaicin and heat. Smart et al. (2000, 2001) found that the endogenous lipid anandamide is a full agonist at the human vanilloid receptor and characterized human vanilloid VR1 pharmacology using FLIPR. With these methods, Gunthorpe et al. (2004) identified and characterized a potent and selective vanilloid receptor (VR1/TRPV1) antagonist.

PROCEDURE

Cloning and Expression of VR1 Receptors in HEK293 Cells

Human VR1 cDNA was identified using the published rat VR1 sequence (GenBank accession AF029310) to search public nucleotide databases. Expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends using cDNA templates from a number of tissue sources. The full cDNA was amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double-strand sequenced, and stably expressed in HEK293 cells. Rat VR1 cDNA was amplified from rat DRG cDNA and similarly expressed in HEK293 cells.

Cell Culture

hVR1-HEK293 cells were grown as monolayers in minimum essential medium (MEM) supplemented with non-essential amino acids, 10% fetal calf serum, and 0.2 mM L-glutamine, and maintained under 95%/5% O₂/CO₂ at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 20. Dissociated rat neonatal DRG cultures were prepared as described by Skaper et al. (1990).

Electrophysiological Studies

Cells were plated and cultured on glass coverslips at 26,000 cells cm⁻² and whole-cell voltage clamp recordings were performed at room temperature (20–24°C), using standard methods. The extra-

cellular solution consisted of (mM): NaCl 130, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 30, HEPES-NaOH 25, pH 7.3. For anandamide application this solution was supplemented with 0.2% lipid-free bovine serum albumin. Patch pipettes of resistance 2–5 MΩ were fabricated on a Sutter Instruments P-87 electrode puller and were filled with the following solution (mM): CsCl 140, MgCl₂ 4, EGTA 10, HEPES-CsOH 10, pH 7.3. All recordings were made from single, well isolated, phase-bright cells. Currents were recorded at a holding potential of –70 mV using an Axopatch 200B amplifier. Data acquisition and analysis were performed using the pClamp7 software suite. Drug applications were affected with an automated fast-switching solution exchange device (Warner Instruments SF-77B; time for solution exchange ≈30 ms).

Measurements of [Ca²⁺]_i Using the FLIPR

hVR1-HEK293 cells were seeded into black-walled clear-base 96-well plates (Costar, UK) at a density of 25,000 cells per well in MEM, supplemented as above, and cultured overnight. The cells were then incubated with MEM containing the cytoplasmic calcium indicator Fluo-3AM (4 μM; Teflabs, Austin, Tex., USA) at 25°C for 120 min. The cells were washed four times with, and finally cultured in, Tyrode's medium containing 0.2% BSA, before being incubated for 30 min at 25°C with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices, UK) to monitor cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 540 nm) (Sullivan et al. 1999) before and after the addition of various agonists.

EVALUATION

Responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean ± SEM unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software, Calif., USA). pK_B values were generated from IC₅₀ curves for the antagonist vs a fixed EC₈₀ concentration of agonist using the Cheng–Prusoff equation.

MODIFICATIONS OF THE METHOD

Rao and Kaminski (2006) reported that induction of intracellular elevation by Δ⁹-tetrahydrocannabinol in T cells involves TRPC1 channels.

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A.1.2**Studies in Isolated Organs****A.1.2.1** **α -Sympatholytic Activity in Isolated Vascular Smooth Muscle****PURPOSE AND RATIONALE**

Noradrenaline and other sympathomimetic drugs increase vascular smooth muscle tone by stimulation of α -adrenergic receptors. Contractions can be antagonized by α -adrenergic receptor blocking agents such as phentolamine. Drugs can be tested for their capacity of reducing vascular smooth muscle contractions induced by the adrenergic receptor-activating agent noradrenaline. Moreover, effects of peptides, such as bradykinin, can be tested with strips of aorta or pulmonary artery.

PROCEDURE

As donor animals Pirbright White guinea pigs of either sex weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 200–300 g are used. The vessels to be tested are the thoracic aorta or the arteria pulmonalis. The animals are sacrificed by stunning and exsanguination. The pulmonary artery or the thoracic aorta is quickly removed and cut into helical strips of 1–2 mm width

and 15–20 mm length. The strips are mounted in an organ bath with a preload of 1 g. Krebs-Henseleit buffer solution containing 11.5 M glucose is maintained at 37°C and oxygenated with 95% O₂, 5% CO₂. Isotonic or isometric registration is performed. Changes in length are recorded isotonicly using a lever transducer (368 type B, Hugo Sachs Elektronik Freiburg). Isometric force is measured with a force transducer (UC-2 Gould-Statham, Oxnard, USA).

Experimental Course

Following an equilibration period of 60 min, contractions are induced by repeated administrations of (–)noradrenaline HCl in concentrations of 2×10^{-6} M for testing the contractions of the pulmonary artery and in concentrations of 2×10^{-8} M for testing the contractions of the aorta. After obtaining a stable plateau of identically sized contractions, cumulative doses of the test compound are added into the organ bath. Consecutive concentrations are given when the response of the previous dose has reached a plateau.

Controls at the end of the experiment: If a compound does not show vasorelaxing activity at any dose, the sensitivity of the preparation is tested by adding phentolamine (1×10^{-7} M).

If a compound shows vasorelaxing activity, the reversibility of the relaxation is tested by increasing the noradrenaline concentration.

EVALUATION

The contractile force is determined before and after drug administration.

Percent inhibition of spasmogen-induced contraction by test drug is calculated as compared to the maximal contraction with a spasmogen alone (= 100%).

IC_{50} values are determined from the individual dose-response curves. IC_{50} is defined as the dose of drug leading to a 50% relaxation of noradrenaline-induced contraction.

MODIFICATIONS OF THE METHOD

The isolated vena cava of rabbits can be used for assaying α -adrenolytic activity. The rabbit is sacrificed by CO₂ anesthesia. The vena cava inferior is removed and cut into strips. The percent inhibition of epinephrine or norepinephrine induced contractions is determined.

The effects of bradykinin and bradykinin antagonists can be tested in isolated guinea pig artery and isolated rabbit aorta which contains predominantly the BK₁-receptor type (Regoli and Barabé 1980; Hock et al. 1991).

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated pulmonary artery. Endothelium-intact pulmonary artery rings were contracted with 3×10^{-8} M phenylephrine and a cumulative dose-response curve of relaxation was constructed.

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A.1.2.2

α -Sympatholytic Activity in the Isolated Guinea Pig Seminal Vesicle

PURPOSE AND RATIONALE

The seminal vesicles of guinea pigs and rats are tubular organs whose longitudinal and annular muscles are innervated by the sympathetic system. The inhibition of contractions induced by norepinephrine or the α_1 -selective agonist phenylephrine indicates α -sympatholytic activity. Sharif and Gokhale (1986) recommended the use the isolated rat seminal vesicle as a rather sensitive and specific model.

PROCEDURE

Male guinea pigs weighing 300 to 600 g are sacrificed by a blow to the neck. Both seminal vesicles are prepared and placed in Ringer's solution in an organ bath maintained at 32°C and being oxygenated with 95% O₂/5% CO₂. Isotonic or isometric registration is performed. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik Freiburg). Isometric force is measured with a force transducer (UC-2 Gould-Statham, Oxnard, USA).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 1 to 5 μ g/ml or phenylephrine HCl in concentrations of 10 to 50 μ g/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three min later, the previous concentration of norepinephrine or phenylephrine is added. As standard phentolamine is used in concentrations of 3 to 30 $\times 10^{-7}$ M.

EVALUATION

Contractions of the seminal vesicle induced by the α -adrenergic agonists after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947)

MODIFICATION OF THE METHOD

Leitch (1954) recommended the use of isolated seminal vesicles of rats for the assay of sympatholytic drugs.

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A.1.2.3

α -Sympatholytic Activity in the Isolated Vas Deferens of the Rat

PURPOSE AND RATIONALE

The vas deferens of the guinea pig or preferably the rat is used for quantitative evaluation of adrenergic antagonists. The response of this organ to α -adrenergic agonists consists of a strong rapid contraction followed

by quick relaxation on washing the agonists out of the tissue.

PROCEDURE

Male Wistar rats weighing about 300 g are used. The animals are sacrificed by a sharp blow to the neck, the vasa deferentia are dissected free from the extraneous tissues and suspended in a organ bath containing Tyrode solution being oxygenated with a 95% O₂ and 5% CO₂ mixture at 32°C. Isotonic registration is performed at a preload of 0.5 g. Changes in length are recorded isotonicly using a lever transducer (e. g., 368 type B, Hugo Sachs Elektronik, Freiburg, FRG).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (-)norepinephrine HCl in concentrations of 0.5, 1.0, 2.0, or 4.0 µg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three min later, the previous concentration of norepinephrine is added. As standard phen-tolamine is used in concentrations of 3 to 30 × 10⁻⁷ M.

EVALUATION

Contractions of the vas deferens induced by the α -adrenergic agonist after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x - values according to Schild (1947).

MODIFICATIONS OF THE METHOD

Electrical stimulation of the isolated ductus deferens results in the release of norepinephrine. Stimulation induced contractions of this organ are inhibited by clonidine which impairs adrenergic neurotransmission by activating inhibitory α -receptors. The ductus deferens is suspended in an organ bath bubbled with carbogen and maintained at 37°C. Tension is adjusted to 25 mN. Following a 45 min equilibration period, supramaximal amplitude stimulation by a HSE type 2 stimulator (Hugo Sachs Elektronik, Freiburg) is applied. After stabilization of the response, clonidine is added to the organ bath in accumulated doses. Test compounds are added 5 min prior to clonidine administration. The percent potentiation of clonidine induced inhibition of contractions is determined.

Taylor et al. (1983) used the rat deferens for pharmacological characterization of purinergic receptors.

Nerve-muscle preparations of the vas deferens have been reviewed by Holman (1975).

Hughes et al. (1974) used the electrically stimulated mouse vas deferens for assessment of the agonistic and antagonistic activities of narcotic analgesic drugs.

Ross et al. (2001) used the mouse vas deferens to study structure-activity relationship for the endogenous cannabinoid, anadamide, and certain of its analogues at vanilloid receptors.

Oka et al. (1980) recommended the vas deferens from rabbits as a specific bioassay for opioid κ -receptor agonists.

Mutafova-Yambolieva and Radmirov (1993) studied the effects of endothelin-1 on electrically- or drug-induced contractile responses mediated by purinergic or adrenergic receptors in the isolated prostatic portion of rat vas deferens.

Ward et al. (1990) used isolated vasa deferentia preparations from rat and mouse to study the pharmacological profile of the analgesic pravadoline.

Hukovic (1961) described an isolated ductus deferens preparation together with the sympathetic hypogastric nerve of the guinea pig.

Cordellini and Sannomiya (1984) pretreated guinea pigs with reserpine. In the isolated vasa deferentia concentration-effects curves to phenylephrine were established in the presence of cocaine. The antagonistic effect of phenoxybenzamine was used for receptor occupancy studies.

Donoso et al. (1992) studied neurotransmission in epididymal and prostatic segments of isolated superfused rat vas deferens preparations.

Vaupel and Su (1987) used the vas deferens preparation of guinea pigs to study sigma and phencyclidine receptors.

Eltze (1988) used the field-stimulated (95% of maximum voltage, 0.1 Hz, 0.5 ms) portion of rabbit vas deferens to study muscarinic M₁ and M₂-receptors.

Dumont et al. (1997) used the isolated guinea pig heart and the isolated rat vas deferens for *in vitro* bioassays of calcitonin gene-related peptide (CGRP) agonists and antagonists.

Poyner et al. (1999) found concentration-dependent inhibitions of the electrically stimulated twitch responses of guinea pig vas deferens by calcitonin gene-related peptide, amylin and adrenomedullin.

Couldwell et al. (1993) found that the rat prostate gland possesses a typical α_1 -adrenoceptor similar to that found in the vas deferens.

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated vas deferens. The prostatic half was

suspended under 0.5 g resting tension and equilibrated in Krebs solution at 37°C. Contractile responses of the prostatic vas were induced by electrical field stimulation at 0.2 Hz, 1.0 ms and 60 V through parallel platinum electrodes either side of the tissue.

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A.1.2.4 α -Sympatholytic Activity in the Isolated Rat Spleen

PURPOSE AND RATIONALE

α -Stimulant agents (such as epinephrine, norepinephrine) or electrical stimulation induce contractions in sympathetically innervated organs such as spleen smooth muscle. These effects can be antagonized by drugs with α -blocking activities such as phentolamine.

PROCEDURE

Male Sprague-Dawley rats weighing 180–220 g are used. The animal is sacrificed in CO₂ anesthesia. The spleen is removed and cut longitudinally into two halves. Each part is placed in an organ bath containing nutritive solution. The bath solution is bubbled with carbogen and maintained at 37°C. Following a 30 min incubation period under a tension of 0.5 g, contractions are elicited by administration of epinephrine (10⁻⁶ g/ml) or norepinephrine 10⁻⁶ g/ml). After obtaining 3 approximately identical spasms, the test compound is administered followed by the addition of the spasmogen 5 min later. The contractile response is allowed to plateau and recorded.

Standard compound:

- phentolamine

EVALUATION

The contractile force is recorded at its maximal level before and after drug administration. The percent inhibition of epinephrine or norepinephrine induced contraction is determined.

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A.1.2.5

α -Sympatholytic Activity in the Isolated Rat Anococcygeus Muscle

PURPOSE AND RATIONALE

The rat anococcygeus muscle as pharmacological tool was introduced by Gillespie (1972, 1980), Gibson and Gillespie (1973). This smooth muscle has a dense adrenergic innervation and contracts to noradrenaline, acetylcholine, 5-hydroxytryptamine, but not to histamine. Moreover, the muscle contracts to field stimulation or stimulation of extrinsic nerves. The preparation can be used to assess the pre- and post-synaptic α -adrenoceptor blocking activity of drugs (Doggrell 1980, 1983).

PROCEDURE

The two anococcygeus muscles arise from the upper coccygeal vertebra close to one another in the midline of the pelvic cavity. The muscles pass caudally, lying first behind and then to one side of the colon, finally joining together to form a ventral bar in front of the colon a few mm from the anus. The extrinsic nerves pass in a branch of the perineal nerve on either side to enter the deep surface of each muscle just before the formation of the ventral bar.

After sacrifice, the abdomen of rats is opened in the mid-line, the pelvis split and the bladder and urethra removed. Care is required in clearing the lower part of the urethra to avoid damage to the ventral bar of muscle, the only region lying ventral to the colon. The colon is then cut through at the pelvic brim, the pelvic portion pulled forward and the delicate connective tissue behind cleared until the anococcygeus muscles come into view. The muscles are isolated, in some instances with the extrinsic nerve intact. The extrinsic nerves on either side run in the posterior scrotal branch of the perineal nerve and leave it to enter the deep surface of the anococcygeal muscles as they lie on the lateral surface of the colon. The ventral bar is cut through and each muscle mounted in a 100 ml bath containing Krebs solution at 36°C. The solution is gassed with 95% O₂+5% CO₂. Tension is measured with isometric transducers and displayed on a polygraph. Field stim-

ulation of the intramural nerves is applied after drawing the muscles through a pair of electrodes similar to those described by Burn and Rand (1960); when the muscles are stimulated through their extrinsic nerves the nerves are drawn through similar electrodes. Stimulation of either intramural or extrinsic nerves is with 1 ms pulses at 20 Hz, and at a supramaximal voltage.

Dose-response curves are established with doses of 2×10^{-7} to 4×10^{-6} M noradrenaline, 4×10^{-7} to 4×10^{-5} M acetylcholine or with graded frequencies of electrical stimulation.

The effect of noradrenaline is abolished by α -adrenergic antagonists, such as 10^{-6} M phentolamine. Dose-response curves show a parallel shift characteristic of competitive antagonism.

EVALUATION

Contractions of the anococcygeus muscle induced by an α -adrenergic agonist after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

MODIFICATIONS OF THE METHOD

Gibson et al. (1990) found L-N^G-nitroarginine to be a potent inhibitor of non-adrenergic, non-cholinergic relaxations in the rat anococcygeus muscle.

Oliveira and Bendhack (1992) found that dopamine has a dual effect in the rat anococcygeus muscle: a partial effect due to an indirect sympathomimetic action and a partial effect due to the interaction with postjunctional receptors.

Brave et al. (1993) investigated the interaction between motor sympathetic and inhibitory non-adrenergic, non-cholinergic nerves in the rat anococcygeus muscle using L-N^G-nitroarginine, an inhibitor of L-arginine:NO synthase.

Cakici et al. (1993) described a coaxial bioassay system consisting of guinea pig trachea as donor organ for epithelial derived-relaxing factors and phenylephrine-precontracted rat anococcygeus muscle as assay tissue.

Iravani and Zar (1993) found differential effects of nifedipine on nerve-mediated and noradrenaline-evoked contractions of rat anococcygeus muscle.

Rand and Li (1993) studied the modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves.

Mudumbi and Leighton (1994) investigated the mechanisms of action of relaxation induced by bradykinin and by electrical field stimulation in isolated rat anococcygeus muscle, where contractile tone had been elevated with clonidine.

Gwee et al. (1995) investigated the prejunctional and postjunctional inhibition of adrenergic transmission in the rat isolated anococcygeus muscle by cimetidine.

Najbar et al. (1996) found that smooth muscle cells in the rat anococcygeus muscle are endowed with two distinct P-2-purinoreceptors which subserve contractions.

De Godoy et al. (2003) evaluated the inhibitory effects of atropine and hexamethonium on the angiotensin-II-induced contraction of rat anococcygeus muscles. Pettibone et al. (1993) examined the inhibitory potency and selectivity of an oxytocin antagonist against oxytocin-stimulated contractions in the **mouse** anococcygeus muscle.

Dehpour et al. (1993) and Radjaee et al. (1996) used isolated anococcygeus muscles from **rabbits** and found an extremely regular activity induced by methoxamine or clonidine.

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A.1.2.6

β_1 -Sympatholytic Activity in Isolated Guinea Pig Atria

PURPOSE AND RATIONALE

The β -agonist isoprenaline (isoproterenol) induces an increase in the frequency and force of contraction of spontaneously beating isolated right atria and potentiates contractions of electrically stimulated isolated left atria. Drugs with β -sympatholytic activity inhibit these isoprenaline-induced effects. β -receptor blocking activity of drugs can be evaluated in isolated right (a) and left (b) guinea pig atria. Since the heart contains predominantly β_1 -adrenoreceptors, β_1 -blocking activity is assessed by this test.

PROCEDURE

Pirbright White guinea pigs of either sex weighing 250–300 g are used. The animal is sacrificed by stunning and exsanguination. The heart is removed, the right or the left atrium is cut off and mounted in a 50 ml organ bath with a preload of 100 mg. The Krebs-

Henseleit solution is maintained at 32°C and aerated with 95% O₂/5% CO₂. Contractions are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik, Freiburg).

Right Atrium

After an equilibration period of 30 min, isoprenaline is administered into the organ bath to potentiate inotropy and frequency of the isolated right atrium. Cumulative doses of isoprenaline are added starting from a concentration of 0.05 µg/ml; consecutive doses are administered at 3 min intervals.

When a stable maximum plateau of the effect is achieved, the organ bath is thoroughly flushed for 1 min; flushing is repeated twice 5 and 20 min later. The whole procedure is repeated with the same isoprenaline concentrations (control baseline values = 100%).

The test compound is then added into the organ bath and 5 min later, again isoprenaline is given at cumulative doses.

If the test compound has β -receptor blocking activity (β -sympatholytic),

1. higher isoprenaline concentrations are necessary to induce the same potentiation of inotropy and frequency or
2. at the same isoprenaline concentrations added as before, the increase in inotropy and frequency is reduced.

At the end of the experiment, again a cycle without test drug is performed.

Left Atrium

The left atrium is stimulated by a square wave stimulator with 2 impulses/s at a voltage of 15 V and an impulse duration of 1 ms. After an equilibration period of 30 min, the β -agonist isoprenaline is added at concentrations of 0.05–0.1 mg/ml. The organ bath is then thoroughly flushed for 1 min. Flushing is repeated twice 5 and 20 min later. The whole procedure is repeated with the same cumulative isoprenaline concentrations (control baseline values = 100%) and flushing procedure.

When a stable plateau of contractions is achieved, the test compound is added into the organ bath and 3 min later, isoprenaline is added again at cumulative concentrations.

If the test compound has β -receptor blocking activity (β -sympatholytic), the isoprenaline-induced effects are inhibited.

In addition, refractory period is determined before and after drug administration.

EVALUATION

a) Percent inhibition of (a) isoprenaline-induced or (b) electrically-induced and isoprenaline-potentiated increased inotropy and frequency by test drug is calculated as compared to pre-drug activity (= 100%).

b) Percent change in refractory period is calculated.

IC_{50} values are determined from the individual dose-response curves.

Statistical evaluation is performed by means of the paired *t*-test.

Standard compounds:

- propranolol HCl,
- amrinon,
- nifedipine,
- and milrinone.

MODIFICATIONS OF THE METHOD

A detailed description of the use of isolated atrial preparations has been given by Levy (1971).

Instead of the right atrium, Doggrell and Hughes (1986) used the isolated right ventricle of the rat for the assessment of the β -adrenoreceptor blocking activity of propranolol and investigated the competitive nature of the isoproterenol antagonism at various doses with Schild-plot analysis. Doggrell (1988) used the isolated left atria of the rat for simultaneous assessment of membrane-stabilizing and β -adrenoreceptor blocking activity.

Berthold et al. (1990) described a method for testing cardiotoxic sodium channel activators in isolated, electrically stimulated left guinea pig atria after potassium depolarization.

Olson et al. (1995) studied the function of isolated rat left atria and papillary muscles and quantified the voltage-response relationship between punctate and field electrical stimulation after pretreatment with reserpine or β -blockers.

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A.1.2.7

β_2 -Sympatholytic Activity in the Isolated Tracheal Chain

PURPOSE AND RATIONALE

Contraction of bronchial smooth muscle is induced by the cholinergic agonist carbachol. The carbachol effect can be antagonized by the β -agonist isoprenaline (isoproterenol). A compound has β -sympatholytic activity if the spasmolytic action of isoprenaline is inhibited. The β -sympatholytic effect of drugs can be evaluated in an *in vitro* model. Since the trachea contains predominantly β_2 -adrenoreceptors, β_2 -blocking activity can be assessed by this test.

PROCEDURE

Male Pirbright White guinea pigs weighing 250–300 g are used. The animals are sacrificed by stunning and exsanguination. The trachea is removed and cut into individual rings. Six rings are connected in series by means of short loops of silk thread. The tracheal chain is mounted in an 50 ml organ bath with a preload of 1 g for isotonic registration. To the nutritive solution (Tyrode) containing ascorbic acid and 1.0 g/L glucose, the α -receptor blocking agent phentolamine (0.1 μ g/ml) and the spasmogen carbachol (80 ng/ml) are added. The solution is maintained at 34°C and aerated with 95% O₂, 5% CO₂

Experimental Course

After an equilibration period of 30 min, cumulative doses of 10⁻¹⁰ to 10⁻⁷ M of the spasmolytic agent isoprenaline are added. When maximal relaxation is obtained, the organ bath is flushed and the procedure repeated. After the two control relaxations with isoprenaline, the tissue is rinsed thoroughly and the first dose of the test compound is administered. Three min later, cumulative doses of isoprenaline are administered as

before. Following a 10 min washout and recovery period, the next dose of the test compound is given. Up to 10 drug concentrations can be tested with one organ.

Standard compounds:

- propranolol
- practolol

EVALUATION

Percent inhibition of isoprenaline-induced relaxation under drug treatment is calculated compared to maximal relaxation induced by isoprenaline alone (control = 100%).

A competitive antagonism of test compound is evaluated and can be quantitated from the dose-response curve.

MODIFICATIONS OF THE METHOD

O'Donnell and Wanstall (1980) used guinea pig tracheal preparations, where K⁺-depolarization was achieved by replacing all the Na⁺ in Krebs solution by an equivalent amount of K⁺ causing a sustained contraction of the preparations. A dose-dependent relaxation effect of isoprenaline could be obtained provided that the preparations were repolarized by washing in normal Krebs solution between curves. pA₂ values were in good agreement with values obtained in other types of tracheal preparations.

Guinea pig superfused trachea and dispersed tracheal cells have been used by Buckner et al. (1995) to compare the effects of isoproterenol and forskolin on immunologic and nonimmunologic histamine release.

Lundblad and Persson (1988) found that epithelial removal is of little consequence for the pharmacology of the guinea pig tracheal open ring preparation *in vitro*.

The rat portal vein has been recommended as model for assessment of β_2 adrenoreceptor blocking activity of drugs by Doggrell (1990).

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A.1.2.8

Angiotensin Converting Enzyme Inhibition in the Isolated Guinea Pig Ileum

PURPOSE AND RATIONALE

The angiotensin-converting enzyme (ACE) is responsible for the formation of the active angiotensin II from the inactive angiotensin I. The same enzyme is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and inhibition of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The guinea pig ileum contracts in response to both peptides, angiotensin II and bradykinin, and can be used for quantitative determination of ACE inhibiting activity.

PROCEDURE

Guinea pigs of either sex weighing 300 to 500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, and the mesentery is being cut away as necessary. When the colon is reached, the intestine is cut free. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode's solution is passed through the tube and the intestine

until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 3 cm length are cut. Preferably, the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into an organ bath with Tyrode's solution at 37°C being oxygenated with O₂. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA). Responses are recorded on a polygraph.

Angiotensin I Antagonism

After an equilibrium time of 30 min, angiotensin I is added in a concentration of 10 ng/ml bath solution. The force of contraction is recorded and the angiotensin I dosage is repeated once or twice until the responses are identical. Then the potential ACE inhibitor is added. After 5 min incubation time, again angiotensin I is added. The contraction is diminished depending on the activity of the ACE inhibitor.

Bradykinin Potentiation

Pieces of guinea pig ileum are prepared as described before. After an equilibrium time of 30 min bradykinin is added in a concentration of 15 ng/ml bath solution. The force of contraction is recorded and bradykinin additions are repeated once or twice until the response is identical. Then the potential ACE inhibitor is added. After 5 min incubation time, again bradykinin is added. The contraction is potentiated depending on the activity of the ACE inhibitor.

EVALUATION

Angiotensin I Antagonism

The contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. Using various doses of the ACE inhibitor *IC*₅₀ values (concentrations inducing 50% inhibition) are calculated. As standards ramipril, enalapril, and captopril are used.

Bradykinin Potentiation

The increase of the contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. As standard ramiprilate is used.

CRITICAL ASSESSMENT OF THE METHOD

The classical method of the isolated guinea pig ileum has been proven to be a reliable method for screening of potential ACE inhibitors.

REFERENCES AND FURTHER READING

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A.1.2.9

Contractile and Relaxing Activity on Isolated Blood Vessels Including Effects of Potassium Channel Openers

PURPOSE AND RATIONALE

The contractile process within the vascular smooth muscle results from an increase in the concentration of intracellular Ca^{2+} . Inhibition of vasoconstriction occurs by addition of calcium antagonists or by removal of extracellular calcium. The vasorelaxing effects of compounds can be tested in isolated rodent arteries (pulmonary artery, thoracic aorta). Arterial rings or strips with or without endothelial lining are contracted with different agents, e. g., extracellular K^+ and Ca^{2+} , the α -adrenoceptor agonists phenylephrine and noradrenaline, the Ca^{2+} ionophore A23187 or the thromboxane receptor agonist U46619. Compounds with vasodilating activity antagonize the induced contractions.

Potassium channel openers such as cromakalin, nicorandil, pinacidil or HOE 234 induce relaxation of contracted smooth musculature (Bolton et al. 1998). These effects are explained by data from patch clamp technique and ion flux experiments as well as by antagonism against potassium channel blockers. They indicate the potential use as antihypertensive and anti-asthmatic drugs (Hamilton and Weston 1989; Edwards and Weston 1990, 1993; Weston and Edwards 1992). The studies are complicated by the high diversity of potassium channels including ATP-sensitive, voltage-sensitive and Ca^{2+} -activated channels (Mourre et al. 1986; Blatz and Magleby 1987; Ashcroft and Ashcroft 1990; Jan and Jab 1990; Pongs 1992; Wann 1993). Since each functional channel appears to consist of four different subunits, the possibility exists that there may be hundreds of different voltage-sensitive K channels, depending on their subunit composition. Ashcroft and Gribble (2000) discussed new windows on the mechanism of action of K_{ATP} channel openers.

Glibenclamide is an antagonist of the ATP-modulated K^+ channel allowing the localization of the binding sites (Eltze 1989; French et al. 1990; Mourre et al. 1990; Miller et al. 1991).

PROCEDURE

Male Pirbright White guinea pigs weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or

Sprague-Dawley rats weighing 250–400 g are used as donor animals. The tested vessels are the thoracic aorta or the arteria pulmonalis.

Materials and solutions

| | Physiological salt solutions (PSS) [mM] | | |
|--------------------------|-----------------------------------------|--------|---------|
| | PSS I | PSS II | PSS III |
| NaCl | 122 | 112 | 72 (92) |
| KCl | 5.0 | 5 | 40 (20) |
| CaCl_2 | 1.2 | – | – |
| MgSO_4 | 0.56 | 0.56 | 0.56 |
| KH_2PO_4 | 1.2 | 1.2 | 1.2 |
| NaHCO_3 | 25 | 25 | 25 |
| EDTA | – | 0.2 | – |
| glucose | 12 | 12 | 12 |

| Contracting agents | |
|---------------------------------------------|-------------------|
| $\text{K}^+ + \text{Ca}^{2+}$ | 40 mM + 0.5 mM |
| $\text{K}^+ + \text{Ca}^{2+}$ | 20 mM + 0.5 mM |
| U 46619 (thromboxane A_2 analogue) | 1 μM |
| A 23187 (calcium ionophore) | 5 μM |
| noradrenaline | 1 μM |
| phenylephrine | 0.1 μM |
| acetylcholine | 1 μM |
| oxyhemoglobin | 10 μM |
| methylene blue | 10 μM |

Animals are sacrificed by stunning and exsanguination. At least 4 isolated organs are tested per drug. The heart and the pulmonary artery are quickly removed and immersed in PSS I at room temperature. The artery is dissected into rings and endothelial cells are removed by gently rubbing the intimal surface. Spirally cut strips of 15–20 mm length and 1–1.5 mm width are suspended at a resting force of 380 mg in an organ bath containing 20 ml oxygenated (95% O_2 , 5% CO_2) PSS I at 37°C. Changes in length are recorded isotonicly using a lever transducer (368 type B, Hugo Sachs Electronic, Freiburg).

To test the effect of compounds on vessels with intact endothelial lining, the thoracic aorta of rats is isolated and dissected free from surrounding tissue. Rings of 3 mm width are cut and suspended in the organ bath containing PSS I. Isometric force is measured with a force transducer (UC-2, Gould-Statham, Oxnard, USA) under a resting tension of 500 mg.

The functional integrity of the endothelium is tested before drug administration. One μM acetylcholine in the organ bath should result in a transient relaxation.

After an equilibration period of 1 h, contraction of each vessel strip or ring is induced by addition of one of the contracting agents into the organ bath.

To induce contractions of potassium-depolarized vessels, three different PSS solutions are used (PSS I for 30 min, PSS II for 3 × 15 min and PSS III). Contraction is induced in the presence of PSS III by adding 0.5 mM Ca²⁺ into the organ bath.

When a stable plateau of contraction is achieved, cumulative concentrations of the test compound are added into the organ bath to obtain drug-response curves. Consecutive concentrations are added either at 1 h-intervals or when the response of the previous dose has reached a steady state level.

In order to study the time course of relaxation and the duration of action, only one concentration is tested.

To test whether the mechanism of action of a vasorelaxing agent is related to the liberation of nitric oxide, methylene blue or oxyhemoglobin (10 μM) are added to the organ bath 15–30 min prior to the cumulative administration of the test compound. Methylene blue or oxyhemoglobin block selectively NO induced relaxation.

EVALUATION

Mean values of relaxation ± SEM are calculated. The height of contraction before the first drug administration is taken as 100%.

IC⁵⁰ values are determined from the individual dose-response curves. IC⁵⁰ is defined as the dose of drug leading to a 50% relaxation of the contraction induced by KCl or other agonists.

Statistical evaluation is performed by means of the *t*-test.

MODIFICATIONS OF THE METHOD

Calderone et al. (1996) compared four **rat** aortic preparation (single ring, spiral strip, zig-zag strip, and multiple ring) on the basis of responses to noradrenaline and acetylcholine. They recommended the multiple ring preparation as the most suitable of all four for the study of vasoactive drugs because of the reproducibility of both contracturant and relaxing responses.

Kent et al. (1982) used rat aortic strips contracted to a stable tension by either phenylephrine or barium chloride for comparison of vasodilators.

Wilson et al. (1988) studied in isolated rings of rat aorta precontracted with noradrenaline the antagonism of glibenclamide against the vasorelaxation induced by cromakalin.

Löhn et al. (2002) cannulated cerebral arteries from mice with glass cannulas on both sides, allowing an application of hydrostatic pressure to the vessel. Diameter was measured by using a videomicroscopic system (Nikon Diaphot, Düsseldorf, Germany) connected to a personal computer with appropriate software for detection of changes of vessel diameter (TSE, Bad Homburg, Germany).

Nishimura and Suzuki (1995) tested the contractile responses to 5-HT in basilar arteries, superior mesenteric arteries and thoracic aortas from **stroke-prone spontaneously hypertensive rats** in comparison to normal Wistar-Kyoto rats and found that that the hyperresponsiveness to 5-HT is mediated by different 5-HT receptor subtypes.

Fouda et al. (1991) used **the isolated tail arteries from rats**. Differences of the vasoconstrictor response to potassium and norepinephrine between tail arteries from spontaneously hypertensive, renovascular hypertensive, and various strains of normotensive rats were found.

Hamilton et al. (1986), Dacquet et al. (1987) studied the effects of calcium entry blockers in **rat portal vein**.

Bråtveit and Helle (1984) studied the inhibition of vascular smooth muscle by vasoactive intestinal peptide (VIP) in the isolated rat portal vein.

Shetty and Weiss (1987) studied the inhibition of spontaneous rhythmic movements and norepinephrine-induced tension responses in the rat portal vein.

Edwards et al. (1991) compared the effects of several potassium-channel openers on rat bladder and rat portal vein *in vitro*.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α-calcitonin gene-related peptide to relax **mesenteric arteries** precontracted with norepinephrine

Chen et al. (1996) studied the contractile effects of noradrenaline and neuropeptide Y given alone or in combination on isolated rat mesenteric resistance vessels.

Gurden et al. (1993) used **guinea pig** aorta relaxation for functional characterization of adenosine receptor types.

Eltze (1989) studied the antagonism of glibenclamide against potassium channel openers in the isolated **guinea pig pulmonary artery**.

Szentmiklósi et al. (1995) used circular segments from the proximal part of the main pulmonary artery of guinea pigs to study contractile and relaxant effects of adenosine receptors.

Pikkers and Hughes (1995) examined the effect of hydrochlorothiazide on intracellular calcium concentration $[Ca^{2+}]_i$ and tone in **guinea pig mesenteric arteries**. Vessels were mounted on a microvascular myograph and loaded with the Ca^{2+} -sensitive fluorescent dye, Fura-2.

Nishimura et al. (1998) used isolated aorta rings from **Syrian hamsters**. Contractile responses were recorded with an isometric transducer (TSE, Bad Homburg, Germany) and stored (TSE Data acquisition software).

Meisheri et al. (1990) recommended the use of the isolated **rabbit mesentery artery** as a sensitive *in vitro* functional assay to detect K^+ -channel-dependent vasodilators.

Mironneau and Gargouil (1979) studied the influence on electrophysiological and mechanical parameters of longitudinal smooth muscle strips isolated from **rabbit portal vein** by means of a double sucrose gap method associated with a photoelectric device for recording contractions.

Lauth et al. (2001) performed superfusion assays with **rabbit jugular vein**. Four venous ring segments (3–4 mm long) were tested simultaneously by mounting them between force transducers and a rigid support for measurement of isometric force. Increasing doses of bradykinin were applied as bolus injections and the ensuing constrictor response was monitored with the aid of a PC-operated analysis System (Biosys, TSE, Bad Homburg, Germany).

McBean et al. (1986, 1988) used isolated segments of the arteria basilaris of **pigs** to detect compounds with antivasoconstrictive properties. Contraction is elicited by $PGF_{2\alpha}$, serotonin or norepinephrine. Specimens are obtained from adult pigs (strain: Deutsche Landrasse) within 30 min after slaughter from the local slaughter house and stored in nutritive solution. The vessels are trimmed to a length of 4 mm, and the segments are suspended between 2 L-shaped metal hooks in a bath containing 20 ml modified Krebs Henseleit solution (NaCl 148 mM, KCl 5.4 mM, $CaCl_2$ 2.2 mM, $NaHCO_3$ 12 mM, glucose 12 mM). The bath solution is maintained at 37°C and continuously gassed with carbogen to produce a resulting pH of 7.35–7.45. The preparation is incubated under a tension of 37.28 mN (optimal passive load producing the largest contractile response to 3×10^{-6} M $PGF_{2\alpha}$). Following a 60 minute stabilization period, the vessels are sensitized with 30 mM KCl for 10 min. The vessels are washed for 1 minute, and allowed to recover for 30 min with additional 1 minute washes at 15 and 30 min. Thereafter, contractions of the vessels are induced by adding

$PGF_{2\alpha}$ at 3×10^{-6} M. The contractile response is allowed to plateau, then the test compound is administered at cumulative doses.

For each test compound a dose-response curve is recorded. The EC_{50} is obtained graphically or by means of a Hill plot. The EC_{50} is defined as the dose of drug producing half maximal response.

Werner et al. (1991) studied the vascular selectivity of calcium antagonists using **porcine** isolated ventricular trabeculae and right **coronary arteries**.

Merkel et al. (1992) used isolated porcine coronary artery rings precontracted with prostaglandin $F_{2\alpha}$ to demonstrate the vasorelaxant activity of an A_1 -selective adenosine agonist.

Miwa et al. (1993) compared the effect of a K^+ -channel opener with cromakalim, nitroglycerin and nifedipine on endothelin-1-induced contraction of porcine coronary artery.

Satoh et al. (1993) investigated in isolated porcine large coronary arteries whether or not the vasorelaxant actions of nicorandil and cromakalim would be selective using seven different vasoconstrictor agonists.

Yokoyama et al. (1994) studied the vasodilating mechanisms of several pyridinecarboximidamide derivatives in isolated porcine coronary arteries.

Makujina et al. (1995) described a procedure that facilitates the eversion of vascular smooth muscle. Vascular segments of porcine coronary artery, approximately 2 cm in length, were sutured to portions of polyethylene tubing inserted into the lumen of the vessel. After being secured and stabilized by the tubing, the vessel was everted while immersed in physiological buffer. Intracellular calcium concentrations (measured by fura-2AM fluorometry) and tension were registered simultaneously in everted rings denuded of endothelium.

Izumi et al. (1996) tested a K^+ -channel opener and related compounds in isolated porcine coronary arteries contracted with 25 mM KCl.

Frøbert et al. (1996) described impedance planimetry as a new catheter-based technique to measure porcine coronary artery pharmacodynamics and compared the results with the commonly used wire-mounted isometric tension technique after *in vitro* application of nifedipine in various concentrations. A four-electrode impedance measuring system was located inside a 12-mm long balloon which was introduced into 3–4 cm long segments of the left anterior descending coronary artery obtained from 70–90-kg Danish Landrace-Yorkshire pigs.

Hamel et al. (1993) dissected segments (3–4 mm long) of temporal ramifications of the middle cerebral

artery from **bovine** brains and mounted them between two L-shaped metal prongs in a tissue bath containing Krebs-Ringer solution at 37°C. Changes in muscle tension were measured by a force displacement transducer and recorded on a polygraph. Several 5-HT receptor agonists were tested for their ability to induce vasoconstriction and their potencies were compared to that of 5-HT. The authors concluded that bovine pial arteries appear to be the best available model for the human cerebrovascular 5-HT_{1D} receptor.

De la Lande et al. (1996) used isolated segments from proximal (4.5 mm i.d.) and distal (0.5 mm i.d.) bovine coronary arteries and found a heterogeneity of response to glyceryl trinitrate.

The isolated **human** coronary artery was used to study the vasoconstriction by acutely acting antimigraine drugs (Saxena et al. 1996a, b, 1997)

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A.1.2.10

Isolated Guinea Pig Ureter

PURPOSE AND RATIONALE

The isolated guinea pig ureter shows phasic-rhythmic contractions after addition of KCl to the organ bath. Inhibition of this effect can be explained as a modulation of potassium channels.

PROCEDURE

Male unfasted guinea pigs weighing 400–500 g are sacrificed and both ureters removed immediately without the part directly connected to the pelvis in order to exclude the pacemaker region responsible for spontaneous activity. Each segment of 2 cm length is

placed in a Petri dish containing Tyrode solution at 37°C, freed of surrounding connective tissue and then suspended at a baseline tension of 0.5 p in a 25 ml organ bath containing Tyrode solution at 37°C being aerated with 5% CO₂/95% O₂, pH 7.4. Contractions are measured isometrically using Gould/Statham UC 2 transducers. After a 15 min equilibration period, KCl is added to the bath in a final concentration of 3×10^{-2} Mol/l and left in the bath for 2 min. KCl induces a constant series of phasic-rhythmic contractions without a rise in baseline tone. Subsequent washing causes the immediate disappearance of the rhythmic contractions. This addition of KCl is repeated and the values of these two experiments are used as initial values. The antagonistic activity is studied by addition of the test drug one min prior to the KCl challenge. Percentage of the following parameters are determined: mean height of contractions, frequency of contractions and the product of mean height and frequency of contractions. For interaction studies the potassium channel blocker glibenclamide 10^{-6} mol/l is added 1 min prior to the test drug.

EVALUATION

Arithmetic means and standard deviations of the data are calculated and compared with initial values using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The isolated guinea pig ureter stimulated with KCl can be used for studies on the modulation of potassium channels.

MODIFICATIONS OF THE METHOD

Yoshida and Kuga (1980) recorded electrical activities in a preparation consisting of the pelvic region and the upper ureter of the guinea pig. Train field stimulation of the pelvic region evoked a train of nerve action potentials followed by a multiphasic smooth muscle action potential after a latency of about 2.5–8.0 s. This smooth muscle response was abolished by tetrodotoxin and dibucaine, and also by cholinergic blocking agents.

The effects of veratridine and of yohimbine on the efflux of norepinephrine from electrically stimulated guinea pig ureters were studied by Kalsner (1992).

Maggi and Giuliani (1994) studied the excitability and refractory period of the guinea pig ureter to electrical field stimulation.

Roza and Laird (1995), Laird and Cervero (1996) studied the pressor responses to distension of the ureter in anesthetized rats as a model of acute visceral pain.

A simple method for measurement of ureteric peristaltic function *in vivo* in anesthetized rats was published by Kontani et al. (1993).

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A.1.2.11

Isolated Corpus Cavernosum

PURPOSE AND RATIONALE

The isolated corpus cavernosum of rabbits has gained interest as pharmacological model since selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (PDE5) were found to be effective in the treatment of erectile dysfunction in man (Ballard et al. 1996; Jeremy et al. 1997; Chuang et al. 1998; Liu et al. 1998; Turko et al. 1999; Wallis 1999; Wallis et al. 1999; Stief 2000; Aydin et al. 2001; Thompson et al. 2001; Lin et al. 2002).

PROCEDURE

Male New Zealand White rabbits weighing 3–4 kg are sedated with an intramuscular injection of 25 mg/kg ketamine+6 mg/kg xylazine. Anesthesia is maintained by intravenous injection of 25 mg/kg nembutal. The penis is removed at the level of the attachment of the corporal bodies to the ischium. The corpus cavernosum (total length about 20 mm) is sharply dissected from tunica albuginea and two longitudinal strips with

unstretched length about 10 mm are made from the proximal, more muscular portion.

Corporal strips are placed in organ baths containing 10 ml Tyrode's buffer (NaCl 124.9 mmol/l, KCl 12.5 mmol/l, MgCl₂·6H₂O 0.5 mmol/l, NaH₂PO₄ H₂O 0.4 mmol/l, CaCl₂ 1.8 mmol/l and glucose 5.5 mmol/l) at 37°C. Each tissue is equilibrated with a mixture of 95% O₂ and 5% CO₂ at pH 7.4. One end of each strip is connected to a force displacement transducer, and changes in muscle tension are measured and recorded with a polygraph. After zeroing and balancing transducers and strip chart, 2.0 g of tension is placed on each strip, and the strips are allowed to equilibrate for 30 min.

Each strip is prestimulated with 10 μM phenylephrine, then relaxed by electrical field stimulation with square wave pulses of 80 V, 1 ms duration at 2–16 Hz frequency. Then sodium nitroprusside (0.01–100 μM) is added as NO donor. Finally, the standard (sildenafil 1 nM to 1 μM) or the test compound is added.

EVALUATION

The dose-dependent increase of relaxation after test compound and standard is measured. From dose-response curves activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Wallis et al. (1999) studied the inhibition of human phosphodiesterases PDE1 to PDE6 by sildenafil in various tissues, such as cardiac ventricle, corpus cavernosum, skeletal muscle and retina.

Park et al. (1997) reported functional characterization of angiotensin II receptors in rabbit corpus cavernosum.

Yildirim et al. (1997) investigated the effects of castration and testosterone on the constricting effect of phenylephrine and endothelium-dependent and -independent relaxing effects of different agonists in the corpus cavernosum of male rabbits.

Liu et al. (1998) analyzed the pharmacological effects of *in vitro* ischemia on rabbit corpus cavernosum.

Gupta et al. (1998) found that activation of G_i-coupled postsynaptic α₂-adrenoceptors causes contraction of smooth muscles in the corpus cavernosum of rabbits.

Teixeira et al. (1998) used a bioassay cascade to study the effect of *Tityus serrulatus scorpion* venom on the rabbit isolated corpus cavernosum.

Cellec and Moncada (1998) used the clitoral corpus cavernosum of female rabbits to study the role of nitroergic neurotransmission in non-adrenergic non-cholinergic relaxation responses.

The isolated corpus cavernosum of **rats** has been used by Tong and Cheng (1997), Gemalmaz et al. (2001), and Wingard et al. (2003), of **mice** by Gocmen et al. (1997) and Mizusawa et al. (2001), of **dogs** by Hayashida et al. (1996), Comiter et al. (1997), of **monkeys** by Okamura et al. (1998), of **horses** by Reccio et al. (1997).

Studies in isolated **human** corpus cavernosum were performed by Holmquist et al. (1992), Bush et al. (1992), Rajfer et al. (1992), Cellec and Moncada (1997), Ballard et al. (1998), Omote (1999), Wallis et al. (1999), Lin et al. (2000), and Stief et al. (1998, 2000).

In vivo studies measuring intracavernous pressure in **rats** were performed by Ari et al. (1996), Chan et al. (1996), Moody et al. (1997), Reilly et al. (1997), Chang et al. (1998), Mills et al. (1998), Gemalmaz et al. (2001), Takagi et al. (2001), Rajasekaran et al. (2005), and Wingard et al. (2006).

Cashen et al. (2002) measured intracavernous pressure in anesthetized mice.

In vivo studies on penile erection were performed in **cats** by Champion et al. (1997).

Intracavernous pressure was measured *in vivo* in anesthetized **dogs** by Ayajiki et al. (1997), Sarikaya et al. (1997), Carter et al. (1998), and Noto et al. (2000).

Bischoff and Schneider (2000) described a simple and quantitative model to study agents that influence penile erection in **conscious rabbits**. Erection was assessed by measuring the length of uncovered mucosa before and after the intravenous administration of agents. Animals did not require anesthesia during the course of the study.

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A.1.3

Cardiovascular Analysis in Vivo

A.1.3.1

Hemodynamic Screening in Anesthetized Rats

PURPOSE AND RATIONALE

The test is used to detect the effect of compounds on blood pressure and heart rate of anesthetized rats and to check for possible interference with adrenergic receptors. Antihypertensive agents with different mechanisms of action can be detected with this test.

PROCEDURE

Male Sprague-Dawley rats weighing 250–400 g are used. At least 2 animals are necessary for screening of one compound. The rats are anesthetized by intraperitoneal injection of 8 ml/kg of a solution of 8% urethane and 0.6% chloralose. The trachea is cannulated to facilitate spontaneous respiration. Body temperature is maintained at 38°C by placing the animal on a heating pad.

The left femoral vein is cannulated for drug administration, which is standardized to injections of 0.2 ml/100 g body weight over a period of 1 min. For measurement of hemodynamic parameters and for intra-arterial administration of test compound, a cannula is inserted retrogradely into the right carotid

artery. The tip of the catheter is positioned close to the origin of the subclavian artery. This allows most of the injected substances to reach the CNS via the vertebral artery before going into the general circulation.

For continuous monitoring of blood pressure (systolic and diastolic pressure) and heart rate, the catheter is connected to a pressure transducer (Statham DB 23).

When stable hemodynamic conditions are achieved for at least 20 min (control values), test boli of adrenaline (1 mg/kg) and isoprenaline (0.25 mg/kg) are administered. When baseline values are again established, increasing doses of the test substance (0.01, 0.1, 3.0 mg/kg) are given intra-arterially. In case of no effect, the interval between successive doses is 15 min, otherwise 60 min. To check for α - or β -blocking activity, adrenaline and isoprenaline administration is repeated after injection of the highest dose of test compound. If the test compound shows no effect, a standard antihypertensive compound is administered for control purpose.

Hemodynamic parameters are recorded continuously during the whole experiment.

EVALUATION

Changes in blood pressure and heart rate after drug administration are compared to control values obtained during the 20 min pre-drug period.

Maximal changes in BP and HR and duration of the effect are reported.

The results are scored relative to the efficacy of standard compounds for the degree of the effect and the duration of the effect.

Statistical significance is not tested because of the small number of animals used ($n=2$, sometimes 3 or 4) but larger numbers of animals have to be used for quantitative evaluation.

CRITICAL ASSESSMENT OF THE METHOD

Due to the administration of the test compounds via the right common carotid artery not only peripherally acting vasodilators and neuron blockers but also compounds affecting the blood pressure regulating mechanisms in the CNS are detected. Bolus injections of adrenaline and isoprenaline reveal possible α - or β -antagonistic effects.

Standard data:

The following compounds at the doses indicated lead to a strong decrease in blood pressure:

- Clonidine 0.008 mg/kg
- Dihydralazine 1.0 mg/kg
- Phentolamine 3.0 mg/kg

- Prazosin 0.1 mg/kg
- Propranolol 1.0 mg/kg
- Urapidil 1.0 mg/kg
- Verapamil 0.1 mg/kg

MODIFICATIONS OF THE METHOD

Several authors (Mervaala et al. 1999; Wallerath et al. 1999; Rothermund et al. 2000; Baltatu et al. 2001) monitored arterial pressure and heart rate using a pressure transducer system and continuously recorded on a computer-based registration system (TSE, Bad Homburg, Germany).

A procedure for differential intra-arterial pressure recordings from different arteries in the rat was described by Pang and Chan (1985).

DeWildt and Sangster (1983) described the evaluation of derived aortic flow parameters measured by means of electromagnetic flowmetry as indices of myocardial contractility in anesthetized rats.

Using a special Millar ultraminiature catheter pressure transducer and a thermodilution microprobe, Zimmer et al. (1987, 1988) measured right ventricular functional parameters in anesthetized, closed-chest rats.

Veelken et al. (1990) published improved methods for baroreceptor investigations in chronically instrumented rats.

Salgado and Krieger (1988), de Abreu and Salgado (1990), Da Silva et al. (1994) studied the function of the **baroreceptor reflex** in thiopental anesthetized rats. The left aortic nerve was isolated and supported by a bipolar stainless steel electrode and carefully insulated with silicone rubber. Carotid pressure was recorded simultaneously with aortic nerve discharges on an oscilloscope and monitored with a loudspeaker.

King et al. (1987) developed a cross circulation technique in rats to distinguish central from peripheral cardiovascular actions of drugs. The right common carotid arteries were ligated, and the left common carotid arteries and left and right external jugular veins of two phenobarbital-anesthetized rats were connected with polyethylene tubing so that peripheral blood from one rat, A, supplied the head of another rat, B, and then returned to the body of A, and vice versa, for peripheral blood from rat B. Each rat was artificially ventilated with O₂, the chest was opened, and both subclavian arteries were ligated. Prior to the ligation of the subclavian arteries, blood flow from rat A supplied its own brain and both brain hemispheres but not the brain stem of rat B. Following subclavian artery ligation, blood flow from rat A did not supply A's brain, but supplied both hemispheres and brain stem of rat B.

The head of each rat was, therefore, rendered dependent on the carotid arterial blood supply from another rat. This rat cross-circulation preparation can be used to separate the central and peripheral cardiovascular actions of drugs.

Zavisca et al. (1994) studied the hypertensive responses to defined electrical and mechanical stimuli in anesthetized rats. Rats were given etomidate, 3.8 mg/kg/h intravenously following carotid artery and jugular vein cannulation. At 15 min after beginning the infusion, 4 types of noxious stimuli were administered sequentially at 1-min intervals: Type 1: Square electrical waves 125 cps, 1.6 ms, 2-s duration, varying current from 0.4 to 12 mA; Type 2: A single 10-mA electrical stimulus, 5-s train duration; Type 3: Tail clamping; Type 4: Skin incision. After each stimulus, maximum change in systolic blood pressure was measured. Graded electrical stimulation allowed the best quantitative evaluation of the hypertensive response to noxious stimuli.

Hyman et al. (1998) described a novel catheterization technique for the *in vivo* measurements of **pulmonary vascular responses** in rats. Male Charles River rats weighing 26–340 g were anesthetized and strapped in supine position to a fluoroscopic table. They breathed air enriched with oxygen through an endotracheal tube inserted by tracheostomy. Catheters were inserted into the femoral blood vessels. The venous catheters were passed to the right atrium under fluoroscopy. A F-1 thermistor catheter was passed from the left carotid artery into the ascending aorta under fluoroscopy, and a PE-50, 150-mm plastic catheter with a specially constructed curved tip was passed fluoroscopically from the left jugular vein into the main pulmonary artery. A plastic radiopaque 22-gauge catheter 100 mm in length with a curved tip was passed with a 0.025 mm soft-tip coronary guiding catheter from the right jugular vein through the right atrium to the inferior vena cava. The coronary soft-tip guide was then withdrawn. A specially curved 102.5-mm transseptal needle, 0.4 mm in diameter, was then passed through the catheter. Both the needle and catheter were withdrawn into the superior portion of the right atrium under fluoroscopic guidance so that the needle and catheter both rotated freely. With the rat in a slight left anterior oblique position, the catheter and needle were carefully rotated anteriorly to the intra-atrial septum. With gentle pressure, the catheter and needle can be felt and seen fluoroscopically to pass through the atrial septum. As the needle was withdrawn, the curve of the catheter permitted passage of the tip into the vein draining either the left or right

lower lobe. The catheter was carefully positioned near the pulmonary venoatrial junction and fixed in place. Mean pressures in the femoral artery, pulmonary artery and pulmonary vein at the venoatrial junction were measured with pressure transducers and recorded on a polygraph. Cardiac output was obtained in triplicate by delivering 0.1 ml normal saline at room temperature into the femoral venous catheter at the right venoatrial junction and determining thermodilution cardiac output with the thermistor catheter in the ascending aorta.

Hayes (1982) described a technique for determining contractility, intraventricular pressure, and heart rate in the **anesthetized guinea pig** by inserting a needle, attached to a pressure transducer, through the chest wall into the left ventricle.

Williams et al. (1995) used **castrated male ferrets** anesthetized by intramuscular injection of a mixture of 55 mg/kg ketamine and 4 mg/kg xylazine to measure the effects of a nonpeptidyl endothelin antagonist on endothelin-induced pressor responses.

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A.1.3.2

Blood Pressure in Pithed Rats

PURPOSE AND RATIONALE

The pithed rat has been proposed for assessing pressor substances by Shipley and Tilden (1947). The preparation is frequently used to evaluate drug action on the cardiovascular system since this preparation is devoid of neurogenic reflex control that may otherwise modulate the primary drug effect.

PROCEDURE

Male rats weighing 250–350 g are prepared for pithing under halothane anesthesia. The left carotid artery is cannulated for blood pressure monitoring and blood sampling. Furthermore, the trachea and the right jugular vein are cannulated. The rats are pithed inserting a steel rod, 2.2 mm in diameter and about 11 cm in length, through the orbit and foramen magnum down the whole length of the spinal canal. Via the tra-

cheotomy tube, the animals are ventilated with a small animal ventilation pump. Inspired air is oxygen-enriched by providing a flow of oxygen across a T-piece attached to the air intake of the ventilation pump (Harvard Apparatus model 680). The rats are ventilated at a frequency of 60 cycles/min with a tidal volume of 2 ml/100 body weight. Thirty min after pithing, a 0.3 ml blood sample is withdrawn from the carotid cannula and immediately analyzed for pO₂, pCO₂, pH, and derived bicarbonate concentration using an automatic blood gas analyzer. By alterations of the respiratory stroke volume of the pump, the values are adjusted to: pCO₂ 30–43 mm Hg, pH 7.36–7.50, pO₂ 87–105 mm Hg.

Continuous registration of blood pressure and cardiac frequency (Hellige He 19 device and Statham P 23 Db transducer) is performed via the left carotid artery.

In order to measure α_1 and α_2 antagonism, first dose-response curves are registered using doses of 0.1–30 $\mu\text{g}/\text{kg}$ i.v. phenylephrine (a selective α_1 agonist), and 1–1000 $\mu\text{g}/\text{kg}$ i.v. BHT 920, (a selective α_2 agonist). The test drug is administered intravenously and the agonist dose-response curves are repeated again 15 min later.

EVALUATION

If the curve of blood pressure response to the agonists is shifted, dose-response curves are plotted on a logarithmic probit scale and potency ratios are calculated.

MODIFICATIONS OF THE METHOD

Gillespie and Muir (1967) described a method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat by coating those parts of the pithing rod which lay in the sacral and cervical region of the spinal cord with high-resistance varnish to restrict stimulation to the thoracolumbar region. The steel rod is insulated with an adhesive throughout its length except for a 5 cm section which provides sufficient a stimulation area of the lower thoracolumbar nerves. For stimulating nerves fibers supplying exclusively the heart, a pithing rod is used which is insulated throughout its length except for a 0.5 cm section 7 cm proximal to the tip. The spinal cord is stimulated electrically using the pithing rod as the cathode and a hypodermic needle which is inserted under the skin near the right hind-limb, as the anode. Varying the intensity and/or the duration of the stimulation, dose-response curves can be registered which are altered after treatment with drugs.

Curtis et al. (1986) described an improved pithed rat method by mounting the preparation vertically with

the head pointing downward resulting in considerably higher blood pressure and heart rate.

MacLean and Hiley (1988) studied the effect of artificial respiratory volume on the cardiovascular responses to an α_1 - and α_2 -adrenoceptor agonist in the air-ventilated pithed rat using microsphere technique and analysis of arterial blood gases and pH.

Trolin (1975) used decerebrated rats to study the clonidine-induced circulatory changes.

Balt et al. (2001) compared the angiotensin II type 1 (AT1) receptor blockers losartan, irbesartan, telmisartan and the ACE inhibitor captopril on inhibition of angiotensin-II-induced facilitation of sympathetic neurotransmission in the pithed rat.

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A.1.3.3

Antihypertensive Vasodilator Activity in Ganglion-Blocked, Angiotensin II Supported Rats

PURPOSE AND RATIONALE

The method is used to demonstrate direct vasodilator activity of potential antihypertensive agents. The experimental model is an anesthetized, ganglion-blocked rat whose blood pressure is maintained by an intravenous infusion of angiotensin II. The test allows to differentiate between centrally acting antihypertensives and peripheral vasodilators.

PROCEDURE

Male Wistar rats weighing 275–450 g are anesthetized with a combination of urethane (800 mg/kg) and chloralose (60 mg/kg) administered intraperitoneally in a volume of 10 ml/kg. Following induction of anesthesia, chlorisondamine (2.5 mg/kg) is injected into the peritoneal cavity to abolish sympathetic and parasympathetic nerve activity. The right femoral artery is cannulated to monitor blood pressure (Statham pressure transducer P23Db) and heart rate. Both femoral veins are cannulated to administer drugs or infuse angiotensin II. The trachea is intubated and animals are allowed to breathe spontaneously. Following a stabilization interval of 10–15 min, angiotensin II is infused at a rate of 0.25 or 3.5 $\mu\text{g}/\text{min}$ in a volume equivalent to 0.05 ml/min (Harvard infusion pump).

After an increase of blood pressure, a new elevated steady-state pressure is established within 15–20 min. Drugs are subsequently injected intravenously over an interval of 3 min in a volume of 2 ml/kg. Mean arterial pressure is recorded on a polygraph at 5, 10, 15, 20 and 30 min after initiation of drug administration. Seven to 9 animals are used for each drug and dose level studied.

α -adrenoreceptor blockade can be determined in ganglion-blocked rats. Pressor responses to graded doses of phenylephrine injected intravenously are

obtained before and 15 min after administration of test compounds. Sufficient concentrations of phenylephrine have to be given to ensure a rise in mean arterial blood pressure of 50 mm Hg or more. Data obtained from 5 or 6 animals are averaged and resultant dose-response curves plotted. The dose of phenylephrine required to elicit a 50 mm Hg increase in mean arterial blood pressure is interpolated from dose response curves.

Standard data:

The following compounds are used as standards and, at the doses indicated, lower mean arterial blood pressure by about 50 mm Hg:

- Cinnarizine 3.0 mg/kg, i.v.
- Hydralazine 1.0 mg/kg, i.v.
- Minoxidil 10.0 mg/kg, i.v.
- Saralazine 0.03 mg/kg, i.v.
- Molsidomine 0.1 mg/kg, i.v.

EVALUATION

Mean values \pm SEM are given for mean arterial blood pressure and heart rate. Changes of these parameters after drug administration are compared to control values obtained immediately before the application of the test compound. Statistical significance is assessed by means of the paired *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

A hypotensive response in this model appears to correlate more closely with antihypertensive activity in DOCA-salt hypertensive rats than does a vasodilator response in the perfused hind limb of anesthetized dogs and allows a distinction between central anti-hypertensive and vasodilators.

MODIFICATIONS OF THE METHOD

Santajuliana et al. (1996) developed a standard ganglionic blockade protocol to assess neurogenic pressor activity in conscious rats. Rats were instrumented with arterial and venous catheters for measurement of arterial pressure and heart rate and for administration of three different ganglionic blockers (trimethaphan, hexamethonium, and chlorisondamine).

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A.1.3.4**Blood Pressure in Conscious Hypertensive Rats (Tail Cuff Method)****PURPOSE AND RATIONALE**

Rats with spontaneous or experimentally induced hypertension are widely used for screening of potentially antihypertensive compounds. The indirect tail cuff method allows the determination of systolic blood pressure according to the following principle: The cuff is quickly inflated to well above suspected systolic blood pressure; the pulse will then be obliterated. Thereafter, pressure in the cuff is slowly released and, as the pressure falls below systolic blood pressure, the pulse will reappear. The method is analogous to sphygmomanometry in human and can be applied not only at the tail of awake rats but also in dogs and small primates. The indirect tail cuff method is widely used to evaluate the influence of antihypertensive drugs in spontaneously and experimentally hypertensive rats.

PROCEDURE

Male spontaneous hypertensive rats (Charles River) weighing 300–350 g or rats with experimentally induced hypertension are used.

Surgical Procedure to Induce Renal Hypertension

Male Sprague-Dawley rats weighing 80–100 g are anesthetized by intraperitoneal injection of 0.8 ml 4% chloralhydrate solution. Both kidneys are exposed retroperitoneally. To induce renal hypertension, a silver clip (0.2 mm diameter, 4 mm length) is placed onto both renal arteries, the kidneys are reposed and the wound is closed by suture.

Within 5–6 weeks, operated animals attain a renal hypertension with a systolic blood pressure (BPs) of 170–200 mm Hg (mean normal physiological BPs for rats is 100 mm Hg). Only animals with a BPs = 180 mm Hg are used for the tests.

Test Procedure

The procedure is the same for spontaneously and experimentally hypertensive rats. Groups of 6 animals are used per dose. The control group receives saline only. To reduce spontaneous variations in blood pressure, animals are adjusted to the experimental cage by bringing them into the restraining cage which is enclosed in a 31–32°C measuring chamber 3–4 times before the start of the experiment for a period of 30–60 min.

To measure blood pressure, a tubular inflatable cuff is placed around the base of the tail and a piezoelectric pulse detector is positioned distal to the cuff. The cuff

is inflated to approximately 300 mm Hg. As the pressure in the cuff is slowly released, the systolic pressure is detected and subsequently recorded on a polygraph.

The test substance is administered intraperitoneally or by gavage once per day over a period of 5 days. The usual screening dose of a new compound is 25 mg/kg. Blood pressure and heart rate measurements are taken at the following times:

day 1: predose and 2 h postdrug

day 3: predose and 2 h postdrug

day 5: predose, 2 h postdrug and 4 h postdrug.

Between measurements, animals are returned to their home cages.

Standard compounds:

- endralazine (3 mg/kg p.o.)
- nifedipine (3 mg/kg p.o.)
- urapidil (5 mg/kg p.o.)

EVALUATION

Mean values in systolic blood pressure before and after drug administration and the duration of the effect are determined. Percent decrease in systolic blood pressure under drug treatment is calculated. Statistical significance is assessed by the Student's *t*-test.

Scores for % decrease in systolic blood pressure and for the duration of the effect are allotted.

CRITICAL ASSESSMENT OF THE METHOD

The indirect tail-cuff method is being used in many laboratories with many modifications of the devices. Pfeffer et al. (1971) found a good correlation between values obtained with the indirect tail-cuff method and values measured directly with indwelling carotid arterial cannulae, whereas Buñag et al. (1971) reported a lack of correlation between direct and indirect measurement of arterial pressure in unanesthetized rats and Patten and Engen (1971) found difficulties to measure accurate systolic values at higher blood pressure. A good correlation between direct blood pressure data from the carotid artery in rats and readings with the tail cuff method was found by Matsuda et al. (1987) who developed a six-channel automatic blood pressure measuring apparatus with a highly sensitive photoelectric sensor for the detection of tail arterial blood flow and a microcomputer system for automatic measurement of systolic blood pressure and heart rate and for data acquisition and processing.

MODIFICATIONS OF THE METHOD

Details of the tail-cuff method in rats have been discussed by Stanton (1971).

Special equipment for measuring blood pressure in rats is commercially available (e. g., TSE GmbH, Bad Homburg, Germany).

Widdop and Li (1997) described a simple versatile method for measuring tail cuff systolic blood pressure in conscious rats. A tail cuff consisting of a metal T-piece tube with latex rubber inside the tube is placed around the tail at the proximal end. A piezoelectric transducer (model MLT1010) is strapped to the ventral surface of the tail to record the pulse signal from the caudal artery and connected directly to a MacLab data-acquisition system ADInstruments Pty Ltd.).

The tail-cuff method for measurement blood pressure has been adapted for dogs, monkeys (Wiester and Iltis 1976), and cats (Mahoney and Brody 1978).

Blood pressure can be measured from the hind leg of the rat using a leg cuff and a photoelectric cell situated at the dorsal surface of the foot (Kersten et al. 1947). When the leg is occluded, the foot swells and the amount of light striking the photocell is reduced. When the pressure in the cuff is released, the arterial blood flow is restored, the increase of foot volume is decreased and the amount of light transversing the paw increases.

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A.1.3.5

Direct Measurement of Blood Pressure in Conscious Rats with Indwelling Catheter

PURPOSE AND RATIONALE

The method first described by Weeks (1960) allows the direct measurement of arterial pressure in conscious rats eliminating the influence of anesthesia on cardiovascular regulation.

PROCEDURE

Preparation of Cannulae

In order to prepare the cannulae 7 cm and 12 cm long pieces are cut from PE 10 and PE 20 tubings respectively. A stylet wire is inserted into the PE 10 tubing and the PE 20 tubing is also slipped over the stylet wire. The ends of the tubings are heated in a current of hot air and fused together. Ridges are made to anchor the cannula in the animal's tissue. In order to make a ridge, the stylet wire is left inside the cannula and the cannula is heated in a fine jet of hot air. When the polyethylene at the point of heating becomes soft, the cannula is pressed slightly and thus a ridge is formed. One ridge is formed at the PE 20 tubing, about 0.5 cm away from the junction with the PE 10 tubing, and 3 more ridges are formed on the PE 20 tubing at a distance of about 1 cm from each other, first one being situated about 3 cm away from the free end of the PE 20 tubing. The stylet wire is then removed from the cannula and the PE 10 portion of the cannula near the junction with the PE 20 tubing is wound around a glass rod with a diameter of 4 mm. Two rounds are made. Then it is dipped in a boiling water bath for about 5 s. When taken out of the bath, the cannula retains its circles, forming a spring-like structure.

Implantation of Cannulae

Male Sprague-Dawley rats weighing about 300 g are used. The rat is anesthetized with 45 mg/kg pentobarbital i.p. The area of the neck and the abdomen are shaved and cleaned with 70% alcohol. The viscera are exposed through a midline abdominal incision. A segment of the abdominal aorta is exposed just above the bifurcation. A trocar is passed through the psoas muscles adjacent to this segment of the aorta, through the muscles of the back and under the skin until it emerges

from the skin of the neck. Then the cannula is inserted into the trocar and the trocar is withdrawn from the body. The end of the cannula thus comes out from the neck, being anchored by silk sutures to the neck skin and to the psoas muscle. The cannula is filled with heparin solution and the end which is projecting out from the neck skin is blocked with a tight fitting stainless steel needle. Then the other end of the cannula is implanted into the aorta. The aorta is wiped with a cotton-tipped applicator stick above the bifurcation, occluded above this segment and punctured with a bent 27 gauge hypodermic needle. The tip of the PE 10 catheter is inserted through the needle and advanced up the aorta. The intestines are replaced and the wound sutured. The rats are allowed to recover for one week.

Measurement of Blood Pressure

The occluding stainless steel needle is removed and the cannula flushed with diluted heparin solution. The rat is placed in a small cage to restrict its movements, even so it is free to move. The cannula is connected to a Statham P 23 Db pressure transducer and blood pressure is recorded on a polygraph. Test drugs or standards are administered either subcutaneously or orally. Recordings are taken before and after administration of drug over a period of 1 h.

EVALUATION

Changes of blood pressure are measured for degree and duration. Five rats are used for each dose and compound. The maximal changes of each group are averaged and compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

Direct measurement of arterial blood pressure in unanesthetized rats originally introduced by Weeks (1960) has become a valuable and widely used tool in cardiovascular research.

MODIFICATIONS OF THE METHOD

A detailed description of a slightly modified Week's method has been given by Stanton (1971).

Improvements of the method for continuous direct recording of arterial blood pressure and heart rate in rats have been described by Buñag et al. (1971), Laffan et al. (1972), Buckingham (1976), Garthoff and Towart (1981), Garthoff (1983). A detailed description of permanent cannulation of the iliolumbar artery was given by Remie et al. (1990).

Wixson et al. (1987) described a technique for chronic catheterization of the carotid artery in the rat.

Prepared cannulas are commercially available (IRC Life Science, Woodland Hills CA).

A newer modification uses the access to the aorta via the common carotid artery (Linz et al. 1992). Rats are prepared under thiopental anesthesia with arterial PE-50 lines (Intramedic from Clay Adams, USA). The lines are introduced into the ascending aorta via the right carotid artery for direct measurement of arterial blood pressure and into the jugular vein for i.v. application of test compounds. Both lines, filled with saline containing heparin, are surfaced on the neck. The animals are allowed to recover for at least 2 days. Blood pressure is monitored through Statham R P23 Db transducers connected to a recording device. During measurements the lines are kept open with counter current saline infusion at a rate of 1 ml/h.

Bao et al. (1991) placed one catheter via the right femoral artery in the abdominal aorta in rats for recording mean arterial pressure and two additional catheters via the left carotid artery into the descending aorta for application of bradykinin and bradykinin antagonists.

Arterial pressure was recorded in unanesthetized rats after induction of severe hypertension by complete ligation of the aorta between the origin of the renal arteries by Sweet and Columbo (1979).

Hilditch et al. (1978) described a device for the direct recording of blood pressure in conscious dogs.

Akrawi and Wiedlund (1987) described a method for chronic portal vein infusion in unrestrained rats. Hepatic drug metabolism can be studied by infusion into the portal vein and blood collection from the femoral vein.

Robineau (1988) described a method for recording electrocardiograms in conscious, unrestrained rats. Electrodes were implanted subcutaneously and a socket connector was sutured on the head of the animal. A flexible cord leading to a swivel collector was linked to an ECG amplifier.

Kurowski et al. (1991) reported on an improved method to implant, maintain, and protect arterial and venous catheters in conscious rats for extended periods of time.

Schenk et al. (1992) measured cardiac left ventricular pressure in conscious rats using a fluid-filled catheter.

Tsui et al. (1991) recommended a reliable technique for chronic carotid arterial catheterization in the rat.

Hagmüller et al. (1992) described a tail-artery cannulation method for the study of blood parameters in freely moving rats.

Liebmann et al. (1995) described an *in vivo* long-term perfusion system which is based on au-

tomated, computer-controlled high-frequency heparin (10 U/ml) flushing of a cannula inserted into the tail artery of freely moving rats.

Santajuliana et al. (1996) used conscious rats instrumented with arterial and venous catheters to assess neurogenic pressor activity after administration of ganglionic blockers.

Rezek and Havlicek (1975) described simple cannula systems for the infusion of experimental substances in chronic, unrestrained animals. A cannula with a removable cap is used for infusions into various parts of the digestive tract. Intravenous infusions can be performed through a closed system cannula which avoids a possible introduction of air into the circulation.

Kimura et al. (1988) described a method for chronic portal venous, aortic, and gastric cannulation to determine portal venous and aortic glucose and lactate levels in conscious rats.

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A.1.3.6

Cannulation Techniques in Rodents

PURPOSE AND RATIONALE

Cardiovascular pharmacology requires special techniques for catheterization and permanent cannulation of vessels. A few methods are described below.

A comprehensive literature survey on methods for vascular access and collection of body fluids from the laboratory rat was written by Cocchetto and Bjornsson (1983).

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A.1.3.6.1

Permanent Cannulation of the Jugular Vein in Rats

PURPOSE AND RATIONALE

Permanent cannulation of the jugular vein in rats in combination with a head attachment apparatus allowing easy connection of cannulae was first introduced by Steffens (1969). Modifications were described by Brown and Hege (1972), Nicolaidis et al. (1974) and

by Dons and Havlik (1986). A detailed description was given by Remie et al. (1990).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2 /halothane. The shaven neck of the animal on the right side is disinfected with chlorhexidine solution. The incision is made just above the right clavicle. Connective and adipose tissue are pushed aside with blunt forceps and the jugular vein is exposed. The external jugular vein is followed and the division into the maxillary vein, the facial and the linguofacial vein identified. The largest vein is chosen and mobilized for a distance of about 5 mm. Small artery forceps are used to clamp the vessel. The vein is then ligated rostral to the clamp with 6-0 silk, and a second ligature is put loosely around the vessel, but not tightened. Using iridectomy scissors, a V-shaped hole is cut in the vein 2 mm rostral from the bifurcation. Prior to its insertion into the vessel, a sterile cannula is connected to a 1 ml syringe filled with a heparinized saline solution. The vessel is dilated by means of a sharp pointed jeweler's forceps, the cannula slit between the legs of the forceps and gently pushed into the vessel until the tip is at the level of the right atrium. Then the forceps is removed, the caudal ligature gently tied, and the rostral ligature used to anchor the cannula to the vessel. The cannula is tunneled to emerge at the top of the head. While the skin in the neck is held firmly, the artery forceps is inserted subcutaneously in caudal direction over a distance of about 3 cm, then turned anti-clockwise in the direction of the incision in the neck. The cannula is grasped with the forceps. Then the forceps is pulled back until the cannula emerges at the crown of the head and closed by a small microvascular clamp. The cannula is slid over the short end of a 20G stainless steel needle bent to a 90° angle. The catheter is flushed with saline and filled with polyethylene/heparin solution. The long end of the L-shaped stainless steel adapter is closed with a piece of heat-sealed PE-tubing and the wounds are closed with sutures.

MODIFICATIONS OF THE METHOD

Hutchaleelaha et al. (1997) described a simple apparatus for serial blood sampling from the external jugular vein which permits simultaneous measurement of locomotor activity in freely moving rats.

REFERENCES AND FURTHER READING

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Nicolaidis S, Rowland N, Meile MJ, Marfaing-Jallat P, Pesez A (1974) A flexible technique for long term infusion in unrestrained rats. *Pharmacol Biochem Behav* 2:131-136

Remie R, van Dongen JJ, Rensema JW (1990) Permanent cannulation of the jugular vein (acc. to Steffens). In: Van Dongen, Remie, Rensema, van Wunnik (eds) *Manual of Microsurgery on the Laboratory Rat*. Elsevier Science Publ., pp 159-169

Steffens AB (1969) A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. *Physiol Behav* 4:833-936

A.1.3.6.2

Permanent Cannulation of the Renal Vein in Rats

PURPOSE AND RATIONALE

A detailed description for permanent cannulation of the renal vein in rats was given by Remie et al. (1990).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2 /halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. This provides an excellent view to the vena cava. At its confluence with the vena cava, the right renal vein is stripped of its adipose tissue and the peritoneum is opened. Using small anatomical forceps the peritoneum is detached from the vena cava by making small spreading movements with the forceps just beneath the peritoneum. Subsequently, the vena cava and the renal vein are mobilized for approximately 1.5 cm, to allow for clamping of the vessel. A four or five fine-stitch purse-string is placed in the vessel at the confluence of the vena cava and the right renal vein. Using a Barraquer needle holder and a cotton-wool stick, the 7-0 silk suture, armed with a BV-1 needle, is guided through the vessel. After each stitch, any bleeding has to be immediately arrested by applying light pressure using a cotton-wool stick. Having completed the suture a single knot is made with the drawstrings. Three microvascular clips are then placed on the vena cava and the renal vein; first the proximal clip on the vena cava, followed by the clip on the renal vein and finally the distal vena cava clip. A small aperture is cut immediately inside the purse-string suture using iridectomy scissors and jeweler's forceps. The cannula, which is filled with a heparinized saline solution, is pushed into the opening as far as possible. Subse-

quently, the purse-string suture is pulled taut and the clip of the renal vein removed, while pushing the cannula further. The proximal clip on the vena cava is now removed as quickly as possible. The patency of the cannula is checked and the drawstrings of the purse-string suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

REFERENCES AND FURTHER READING

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A.1.3.6.3

Permanent Cannulation of the Portal Vein in Rats

PURPOSE AND RATIONALE

Several techniques have been described for cannulation of the portal vein in rats (Hyun et al. 1967; Pelzmann and Havemeyer 1971; Suzuki et al. 1973; Sable-Amplis and Abadie 1973; Helman et al. 1984). A detailed description for permanent cannulation of the portal vein in rats was given by Remie et al. (1990). After additional application of platinum electrodes around the portal vein in close proximity to the catheter tip, this model can also be used to study the presynaptic regulation of neurotransmitter release from nonadrenergic nerve terminals (Remie and Zaagsma 1986; Remie et al. 1988, 1989).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2 /halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. Using a micro needle holder and a cotton-wool stick, a four or five, fine stitch purse-string suture (7-0 silk suture armed with a BV-1 needle) is placed in the wall of the portal vein at the side opposite the gastroduodenal vein. The diameter of the purse-string should be about 1 mm. After the suture has been completed a single knot is made with the drawstrings. The portal vein is clamped with a small curved hemostatic bulldog clamp. Using iridectomy scissors and a pair of jeweler's forceps the center of the purse-string is cut, a cannula filled with heparinized saline is inserted into the vessel and pushed upwards. The purse-string is gently tightened taking care not to

obstruct the cannula. The drawstrings of the suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

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A.1.3.6.4

Permanent Cannulation of the Thoracic Duct in Rats

PURPOSE AND RATIONALE

Collection of lymph is rather difficult and has been performed mainly in dogs (Biedl and Offer 1907; Gryaznova 1962, 1963; Vogel 1963). Some techniques have been described for the rat (Bollman et al. 1948; Girardet 1975). Remie et al. (1990) did not obstruct the duct by placing a purse-string suture in the wall of the duct, by which the cannula is secured. The animal's lymph can be collected during the experiment, and after refilling the cannula the lymph flow remains undisturbed.

PROCEDURE

Rats are anesthetized with N_2O_2/O_2 /halothane. After opening the abdominal cavity, the intestines are placed

in gauze moistened with warm saline and laid to the left of the animal. The suprarenal abdominal artery is located and mobilized by gently tearing the connective tissue. Using blunt dissection technique, the thoracic duct is mobilized along the dorsolateral surface of the aorta. A small three to four fine stitch purse-string suture is placed in the wall of the duct, using a 9-0 Ethilon suture. A hole is cut inside the purse-string with a very fine pair of scissors, while holding the wall with angled jeweler's forceps. The cannula which is filled with heparinized saline solution and is inserted into the duct using anatomical forceps. After the tip of the cannula has been inserted into the thoracic duct, the curved forceps are removed and the total tip is pushed into the duct. The ligature is then closed and some lymph will flow into the cannula. The cannula is secured within the abdominal cavity by attaching it to the abdominal muscle near the xiphoid cartilage with a 7-0 silk suture. Following the closure of the abdominal wall and the tunneling of the cannula to the crown of the head, a L-shaped adapter is placed on the cannula, filled with PVP-solution and closed with a heat-sealed polyethylene cap.

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A.1.3.6.5

Portacaval Anastomosis in Rats

PURPOSE AND RATIONALE

In 1877 the Russian surgeon Eck reported the achievement of successful portacaval shunts in dogs. Lee and Fischer (1961), Funovics et al. (1975), de Boer et al. (1986), described portacaval shunt in the rat. A detailed description of surgery for portacaval anastomosis in rats was given by van Dongen et al. (1990).

PROCEDURE

Rats are anesthetized with N₂O₂/O₂ in combination with either enflurane, methoxyflurane or isoflurane. After opening the abdominal wall, the intestines are placed left to the animal on gauze moistened with warm saline solution. Proximally and distally to the animal's right renal vein, the vena cava is then stripped of its adipose and connective tissue, and the retroperitoneal cavity is opened. Using anatomical forceps the peritoneum is dissected from the vena cava by making small spreading movements with the forceps just above the vena cava. The portal vein is pulled slightly to the left using straight anatomical forceps and freed from the hepatic artery and the gastroduodenal artery with curved anatomical forceps. Rostral to the celiac artery, the abdominal artery which is covered with peritoneum is freed from its lateral muscle bed over a length of approximately 5 mm providing enough space for placing a small bulldog clamp at a later stage of the operation. Without occlusion a six fine-stitch purse-string is placed in the wall of the vena cava close to its confluence with the right renal vein. Using a Barraquer needle holder and a cotton wool stick, the 7-0 silk suture armed with a BV-1 needle is guided through the vessel. After each stitch, bleeding has to be arrested immediately, by applying light pressure on the area, again using the cotton wool stick. After the suture has been completed, a single knot is made with the drawstrings. The drawstrings should come together at the rostral part of the purse-string. A bulldog clamp, modified to resemble a Satinsky vascular clamp, is then placed on the vena cava.

Before clamping the abdominal aorta rostral to the celiac artery with a small bulldog clamp, a ligature (7-0 silk) is placed around the portal vein as close as possible to the hilus of the liver. Subsequently, the clamp is placed on the aorta and the ligature tightened. A Heifetz clip is then placed transversely onto the portal vein at its confluence with the gastroduodenal vein. The portal vein is cut just distally from the ligature. A prepared button is slipped over the left-hand straight small anatomical forceps, while the right-hand forceps are used to pass the portal vein to the left-hand anatomical forceps. The vein is then grasped and pulled through the button. Subsequently, the button is pushed as close as possible to the Heifetz clip, and clamped to the clip using a Pilling bulldog clamp.

Using small straight, and curved anatomical forceps the portal vein is reversed around the button and fixed with a previously prepared 7-0 silk suture. The Pilling bulldog clamp is then removed and replaced at the end of the Satinsky clamp for reasons of stability. The vena

cava is then somewhat elevated, bringing it into closer contact with the portal vein button.

A longitudinal cut is made in the purse-string suture using iridectomy scissors and jeweler's forceps. One drawstring of the suture is clamped with a small hemostat and put under slight tension in a rostral direction. The button manipulated by its grip, is pushed into the vena cava. The purse-string is tightened with the left hand whilst the right hand still holds the button in position. The button is released and two additional knots tied. The Satinsky clamp is removed first followed by the Heifetz clamp and the bulldog clamp on the aorta. After replacing the intestines the abdominal wall is closed in two layers.

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A.1.3.7

Cardiovascular Analysis in Anesthetized Mice

PURPOSE AND RATIONALE

To fully utilize the potential of mouse models with specific gene mutations, it is necessary to study the functional consequences of genetic manipulations in fully intact mice. Lorenz and Robbins (1997) developed and validated a methodology to study cardiovascular parameters in closed-chest mice.

PROCEDURE

Adult mice of either sex weighing 25–35 g are anesthetized by intraperitoneal injection of 50 mg/kg ketamine and 100 mg/kg thiobutabarbital. After the mice are placed on a thermally controlled surgical table with body temperature continually monitored via a rectal probe, a tracheotomy is performed with a short length (<1 cm) of PE-90 tubing. The right femoral artery is then cannulated with polyethylene tubing which is pulled over a flame to a small diameter (~0.4 mm OD). The catheter is advanced ~1 cm, near the level of the aorta, and connected directly to a low-compli-

ance COBE CDXIII fixed-dome pressure transducer for the measurement of arterial blood pressure. The right femoral vein is then cannulated with the same type of small-diameter tubing and connected to a microinjection pump for the infusion of experimental drugs. To assess myocardial performance, the right carotid artery is cannulated with a 2F Millar MIKRO-TIP transducer (Model SPR-407, Millar Instruments, Houston TX). This high-fidelity transducer, which has a tip diameter of ~0.67 mm, has a reported frequency response that is flat up to 10,000 Hz and therefore can be used to accurately monitor the high frequency of the mouse ventricular pulse pressure. During continual monitoring of the blood pressure wave to ascertain the anatomic position of the catheter, the tip of the transducer is carefully advanced through the ascending aorta and into the left ventricle. When the stable waveform of the ventricular pressure profile is achieved, the transducer is anchored in place with 7–0 silk sutures. After completion of the surgery, all wounds are closed with cyanoacrylate to minimize evaporative loss of fluid, and the animals are allowed to stabilize for 30–45 min.

EVALUATION

Blood pressure signals from the COBE transducer and from the Millar transducer are amplified and the output is recorded and analyzed with a MacLab 4/s data acquisition system connected to a Macintosh 7100/80 computer which allows the calculation of the following parameters:

- dP/dt first derivative of the ventricular pressure wave,
- MAP mean arterial pressure,
- HR heart rate,
- LVP systolic and diastolic left ventricular pressure,
- $LVEDP$ left ventricular enddiastolic pressure.

Further indices of ventricular performance can be calculated from dP/dt .

MODIFICATIONS OF THE METHOD

Champion et al. (2000) described a **right-heart catheterization technique** for *in vivo* measurement of vascular responses in lungs of intact mice. CD1 mice weighing 25–38 g were anesthetized with thiopentobarbital (85–95 mg/kg i.p.) and ketamine 3 mg/kg i.p.) and were strapped in supine position to a thermoregulated fluoroscopic table. The trachea was cannulated and the animals breathed with room air enriched with 95% O₂/5% CO₂. A femoral artery was cannulated

for the measurement of systemic arterial pressure. Heart rate was electronically monitored from the systolic pressure pulses with a tachometer (Grass model 7P44A). The left jugular vein was cannulated for the administration of agonists and antagonists.

For measuring pulmonary arterial pressure, a special single lumen catheter was constructed. The catheter was 145 mm in length and 0.25 mm in outer diameter, with a specially curved tip to facilitate passage through the right heart, main pulmonary artery, and the left or right pulmonary artery. Before the catheter was introduced, the catheter curve was initially straightened with a 0.010-in. straight angioplastic guide wire to facilitate passage from the right jugular vein into the right atrium at the tricuspid valve under fluoroscopic guidance. As the straight wire was removed, the natural curve facilitated entry of the catheter into the right ventricle. A 0.010-in. soft-tip coronary artery guide wire was then inserted, and the catheter was passed over the guide wire into the main pulmonary artery under fluoroscopic guidance. Pressure in the main pulmonary artery was measured with a pressure transducer, and mean pulmonary artery pressure was derived electronically and recorded continuously.

Cardiac output was measured by the thermodilution technique. A known volume (20 μ l plus catheter dead space) of 0.9% NaCl solution at 23°C was injected into the right atrium, and changes in blood temperature were measured at the root of the aorta. A cardiac output computer equipped with a small-animal interface was used. The thermistor microprobe was inserted into the right carotid artery and advanced to the aortic arch, where changes in aortic blood temperature were measured. A catheter placed in the right jugular vein was advanced to the right atrium or main pulmonary artery for rapid bolus injection of saline. The saline solution was injected with a constant-rate syringe to ensure rapid and repeatable injection of the saline indicator solution. Thermodilution curves were recorded on a chart recorder and pulmonary and systemic blood pressure monitored continuously. Catheter placement was verified by postmortem examination.

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A.1.3.8

Blood Pressure in Anesthetized Cats

PURPOSE AND RATIONALE

Cats are the most sensitive species of cardiovascular regulation. They were used extensively for cardiovascular screening. Recently, experiments in dogs are preferred since this species can be bred more easily in homogeneous strains.

PROCEDURE

Adult cats of either sex weighing 2.5 to 4 kg are anesthetized by intraperitoneal injection of 35 mg/kg pentobarbital sodium. Tracheotomy is performed and a tracheal cannula is inserted so that the cat can be mechanically ventilated with room air. A femoral artery and two femoral veins are cannulated for measurement of arterial blood pressure and systemic administration of drugs. The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler. Rectal temperature is monitored and maintained between 37°C and 38°C with a heating pad.

The following drugs are injected i.v. as challenges:

- epinephrine 0.1, 0.3, 0.5 μ g/kg,
- norepinephrine 0.1, 0.3, 0.5 μ g/kg,
- isoproterenol 0.1, 0.2, 0.4 μ g/kg,
- carbachol 0.1, 0.2, 0.5 μ g/kg.

At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline.

Test drugs are injected at various doses followed by injections of the challenging drugs.

EVALUATION

Dose-response curves of challenging drugs are established before and after injections of the test drugs.

CRITICAL ASSESSMENT OF THE METHOD

Blood pressure experiments in anesthetized cats are very valuable as screening techniques for cardiovascular agents. Moreover, potentiation of norepinephrine response has been used as screening procedure for antidepressants with norepinephrine uptake inhibiting activity.

MODIFICATIONS OF THE METHOD

Sander (1965) investigated the vasoconstrictor and vasodilator effects of procaine in spinal cats. The animals

were anesthetized with ether and ventilated with a positive pressure pump via a tracheal cannula. The spinal cord was then cut between the second and third vertebrae, and ether administration stopped. The remaining portion of the spinal cord above the transection was destroyed by passing a curette through the spinal canal.

Yardley et al. (1989) studied cardiovascular parameters in spinal cats. The animals were anesthetized with 80 mg/kg intravenously administered α -chloralose. The spinal cord was transected or crushed at the first cervical segment after tetracaine hydrochloride (0.125 mg in 0.1 ml) had been injected into this region of the cord. Systemic blood pressure was supported at a level sufficient to maintain constricted pupils (mean value 45 ± 5 mm Hg) by volume expansion with blood from a donor (10–20 ml) or an infusion of dextran.

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A.1.3.9

Cardiovascular Drug Challenging Experiments in Anesthetized Dogs

PURPOSE AND RATIONALE

Sympathomimetic and cholinomimetic compounds as well as angiotensin II and carotid occlusion exert characteristic responses in blood pressure of anesthetized dogs. Antagonism or potentiation of these responses

allow to characterize the cardiovascular activity of a new compound.

PROCEDURE

Adult Beagle dogs of either sex weighing between 8 and 15 kg are anesthetized with 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital and 75 mg/kg sodium pentobarbital. Additional doses of sodium pentobarbital are given as needed. The dogs are intubated with a cuffed endotracheal tube and placed on a Harvard respirator (20 ml/kg, 10–15 cycles/min). A femoral vein and artery are cannulated using polyethylene tubing for drug administration and determination of arterial blood pressure, respectively. The animals are bilaterally vagotomized.

The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiograph connected to a voltage/pressure-pulse coupler.

Drug challenges: One of the following combinations of drugs is administered i.v. to the dogs. The challenges are given in a fixed order: at least twice prior to test drug administration to insure consistent responses and again starting 15 min post test drug. Epinephrine and norepinephrine (1 μ g/kg), isoproterenol (0.25 μ g/kg), carbachol (0.25 μ g/kg), tyramine (100 μ g/kg) are used; bilateral carotid occlusion (45 s), phenylephrine (10 μ g/kg), isoproterenol (0.25 μ g/kg) angiotensin II (0.2 μ g/kg) and carbachol (0.25 μ g/kg) for cardiovascular drugs. At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline. Challenge drug doses are sometimes varied to keep the mean arterial pressure within the following limits: epinephrine (+30 to +60 mm Hg), norepinephrine (–30 to +70 mm Hg), tyramine (+30 to +70 mm Hg), isoproterenol (–30 to –50 mm Hg), carbachol (–30 to –50 mm Hg), phenylephrine (–30 to +70 mm Hg), angiotensin II (+30 to +50 mm Hg), and bilateral carotid occlusion (+30 to +70 mm Hg).

EVALUATION

The recordings are studied to detect any changes in the arterial pressure response to the challenge drug before and after test-drug administration and to observe any changes in blood pressure and heart rate. Results are expressed as the percentage change from the predrug response.

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A.1.3.10

Hemodynamic Analysis in Anaesthetized Dogs

PURPOSE AND RATIONALE

The hemodynamic effects of compounds supposed to affect the cardiovascular system are evaluated by measuring preload and afterload of the heart, contractility, heart rate, cardiac output and peripheral or coronary flow. To measure these cardiovascular parameters accurately, the use of larger animals such as dogs or pigs is necessary.

This experimental model allows the classification of test drugs according to their action as having:

- positive inotropic effects
- negative inotropic effects (Ca²⁺-antagonist, anti-arrhythmic?)
- hypertensive effects
- hypotensive effects
- coronary-dilating effects
- β -blocking effects
- α -blocking effects
- anti-anginal effects
- peripheral-vasodilating effects

PROCEDURE

Male or female inbred Beagle or Labrador-Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, and continued with an infusion of 4–6 mg/kg/h. A catheter is placed into the cephalic vein for intravenous injections. Another catheter is placed into the duodenum for enteral administration. Respiration is maintained with room air through a tracheal tube using a positive pressure respirator, e. g., Bird-Mark-7-respirator. Blood gas analyses are performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannula inserted into the left femoral artery and connected to a Statham pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left common carotid

artery into the left ventricle. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dp/dt_{\max} is differentiated and heart rate is counted. The LVP-signal also triggers a cardiometer.

Cardiac output, pulmonary artery pressure (PAP) and stroke volume are measured by a thermodilution technique using a Cardiac Output Computer (Gould/Statham SP 1245) and a balloon-tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

Myocardial oxygen consumption (MVO₂) is calculated as pressure-work-index according to Rooke and Feigl (1982).

Femoral blood flow and coronary flow are measured with electromagnetic flow probes attached to the femoral artery and the circumflex branch of the left coronary artery (LCX), respectively.

Experimental Course

When stable hemodynamic conditions and blood gas values of pO₂ > 100 mm Hg and pCO₂ < 35 mm Hg are achieved for at least 20 min (control values), the test substance is administered through a catheter inserted into a cephalic vein in doses of 0.1, 0.3, 1.0, and 3.0 mg/kg or into the duodenum in doses of 0.3, 1.0, 3.0, and 10.0 mg/kg.

All parameters are recorded continuously during the whole experiment.

Characteristics

- blood pressure
 - systolic, BPs
 - diastolic, BPd
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- maximal rate of pressure rise, dp/dt_{\max}
- heart rate, HR
- peripheral blood flow in A. femoralis, PF
- blood pressure A. pulmonalis, PAP
- coronary flow, CF
- cardiac output, CO
- stroke volume, SV
- total peripheral resistance, TPR
- left ventricular stroke work, LVSW
- left ventricular minute work, LVMW
- left ventricular myocardial oxygen consumption, MVO₂

CALCULATION OF RESULTS AND EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formulae

- stroke volume [ml/beat],

$$SV = \frac{CO}{HR}$$

- total peripheral resistance [dyns/cm⁵],

$$TPR = \frac{BPm}{CO} \times 79.9$$

- left ventricle stroke work [J/beat],

$$LVS\!W = (BPm - LVEDP) \times SV \times 0.333 \times 10^{-3}$$

- left ventricular minute work [J/min],

$$LVMW = LVS\!W \times HR$$

- left ventricular myocardial oxygen consumption [ml O₂/min/100 g],

$$MVO_2 = K_1(BPs \times HR) + K_2 \times \frac{(0.8BPs + 0.2BPd) \times HR + SV}{BW} + 1.43$$

$$K_1 = 4.08 \times 10^{-4}$$

$$K_2 = 3.25 \times 10^{-4}$$

BPs = systolic blood pressure [mm Hg]

BPd = diastolic blood pressure [mm Hg]

BPm = mean blood pressure [mm Hg]

HR = heart rate [beats/min]

CO = cardiac output [ml/min]

SV = stroke volume [ml/beat]

LVEDP = left ventricular enddiastolic pressure [mm Hg]

BW = body weight [kg]

Changes in parameters measured after drug administration are compared to control values obtained during the 20 min pre-drug period.

Results are presented as mean ± SEM with *n* > 3.

Statistical significance is assessed by means of the paired *t*-test.

MODIFICATIONS OF THE METHOD

The effect of drugs on the carotid artery occlusion effect can be studied in anesthetized dogs. The occlusion of right and left common carotid arteries is performed by squeezing them between a polyethylene tubing and a twine which is passed inside the tubing and around the carotid artery. An occlusion of the carotid arteries for 30 s causes an increase of systolic blood pressure by 40–50 mm Hg. Inhibition of this effect by drugs is tested.

Studies in anesthetized dogs can be used to determine the influence of cardiotonic drugs on propranolol induced cardiac insufficiency (Rajagopalan et al. 1993)

Instead of dogs, **pigs (German landrace)** weighing between 20–35 kg can be used. They are pre-treated with ketamine 500 mg/5 ml i.m., methomidate hydrochloride 200 mg/4 ml i.p., xylazine 60 mg/3 ml i.m., and anaesthetized with 15–20 mg/kg pentobarbital sodium, followed by continuous infusion of 12 mg/kg/h. The parameters are evaluated similarly to the experiments in dogs.

Measurement of cardiac output by the thermodilution method in **rats** was described by Richardson et al. (1962) and Müller and Mannesmann (1981).

Thermodilution methods were used by Rosas et al. (1964) in anesthetized rats, by Carbonell et al. (1985) and by Salyers et al. (1988) in conscious rats to determine hemodynamic parameters.

Oxygen pressure, carbon dioxide pressure and pH in coronary venous and common carotid arterial blood of anesthetized dogs has been measured using a blood gas analyzer (Aisaka et al. 1988).

Acute ischemic left ventricular failure can be induced in anesthetized dogs by repeated injections of plastic microspheres into the left coronary artery (Smiseth and Mjøs 1982; Sweet et al. 1984; Schölkens et al. 1986). A coronary catheter was introduced through the right femoral artery and advanced under fluoroscopy to the left coronary ostium, guided by injection of small amounts of contrast medium. After reaching baseline values, acute left ventricular failure was induced by subsequent intra-coronary injections of plastic microspheres (52.9 ± 2.48 μm non-radioactive tracer microspheres). The microspheres were suspended in saline with a drop of Tween 80 and sonified before use, 1 mg microspheres/1 ml saline corresponding to approximately 12,000 microspheres. (13–16 injections of microspheres or 3.4–5.0 mg/kg). Microspheres were injected every 5 min for 70–90 min. Each microsphere injection effected an immediate and stepwise increase in LVEDP. With this procedure, LVEDP can be increased to a desired level in a very controlled manner. In the 30 min following embolization, LVEDP continued to increase by approximately 5 mm Hg. Animal with arrhythmias had to be excluded from the study. Thirty min after the end of embolization, when hemodynamic parameters had stabilized, drug administrations were started.

Valdes-Cruz et al. (1984) developed an **open-chest preparation in dogs** to validate the accuracy of a two-dimensional Doppler echocardiographic method for

estimating pressure drops across discrete stenotic obstructions.

In order to assess the potential of a single breath technique (using freon-22) as an effective way to estimate cardiac output non-invasively, Franks et al. (1990) measured simultaneously with the single breath technique the aortic flow using an electromagnetic flowmeter in anesthetized dogs.

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A.1.3.11

Hemodynamic Measurements in Conscious Dogs

PURPOSE AND RATIONALE

The potency of a cardiovascular drug depends on the direct effects at the cellular level and on the response of the cardiovascular control mechanisms. The latter are often markedly influenced by anesthesia. The chronically instrumented conscious dog with renal hypertension is therefore a more realistic test model to evaluate the effects of antihypertensive, anti-anginal and cardiotoxic compounds. The test is used to evaluate hemodynamic drug effects in conscious dogs, an experimental model with chronic arterial and ventricular catheterization and renal artery constriction.

PROCEDURE

Male or female Labrador-Setter dogs weighing 15–25 kg are used. They are anaesthetized with 1 mg/kg xylazine i.m., followed by 1 mg/kg xylazine i.v. and 18 mg/kg pentobarbital sodium i.v. For chronic instrumentation and induction of renal hypertension, fluid-filled catheters are implanted into the abdominal aorta and into the left ventricle. The catheters are tunneled subcutaneously and exteriorized on the nape of the neck dorsally. Renal hypertension is induced by placing silastic constrictors around both renal arteries. Hemodynamic measurements are performed after a two-week recovery period or later.

To familiarize the dogs to the test surroundings, they are brought into the laboratory 2–3 times before the start of the study. Thus, drug testing is possible without sedation. During the experiment the animal rests quietly on a laboratory table.

Experimental Protocol

Hemodynamic measurements are performed by connecting the two implanted catheters to Statham pressure transducers. Pressure signals, electronically dif-

ferentiated LVP dp/dt max and heart rate are recorded with a polygraph.

After reaching stable hemodynamic conditions for at least 20 min (control baseline values), the test compound is administered either orally in a gelatin capsule or by intravenous injection into the cephalic vein.

Hemodynamic parameters are recorded continuously starting 30 min before to 120 min after drug administration, and thereafter at 1 h intervals until 6 h after dosage.

EVALUATION

The following parameters are monitored:

- systolic blood pressure [mm Hg]
- diastolic blood pressure [mm Hg]
- left ventricular enddiastolic blood pressure, LVEDP [mm Hg]
- left ventricular pressure at dp/dt max [mm Hg/s]
- heart rate [beats/min]

Mean values \pm SEM are calculated with $n > 3$ as differences to pre-drug control values.

MODIFICATIONS OF THE METHOD

Mann et al. (1987) described a simple procedure for direct blood pressure measurement in conscious dogs using the Vascular-Access-Port™, consisting of a 33 × 13 mm reservoir body affixed to a silicon rubber catheter.

Müller-Schweinitzer (1984) described a method for the assessment of vasoconstrictor agents by recording venous compliance in the conscious dog. Changes in the diameter of the canine saphenous vein, produced by inflation to 45 mm Hg of a sphygmomanometer cuff placed on the upper hind leg, were recorded.

Hintze and Vatner (1983) compared the effects of nifedipine and nitroglycerin in conscious dogs, instrumented for instantaneous and continuous measurements of coronary arterial and left ventricular diameters with an ultrasonic dimension gauge, arterial and left ventricular pressure with implanted miniature gauges, and coronary blood flow with an electromagnetic flowmeter or a Doppler ultrasonic flowmeter.

Shimshak et al. (1986) studied the recovery of regional myocardial contractile function after a 10 min coronary artery occlusion in chronically instrumented conscious dogs.

Wright et al. (1987) described a minimally invasive technique which allows assessment of histamine H₁-receptor antagonist activity in conscious dogs based on the inhibition of tachycardia caused by intravenous ad-

ministration of the H₁-receptor agonist, 2-pyridylethylamine.

Hashimoto et al. (1991) studied the coronary effects of nicorandil in comparison with nitroglycerin in chronic conscious dogs instrumented with ultrasonic crystals and electromagnetic flowmeters in the circumflex coronary artery.

Hartman and Warltier (1990) described a model of multivessel coronary artery disease using conscious, chronically instrumented dogs. A hydraulic occluder and Ameroid constrictor were implanted around the left anterior descending and the left circumflex coronary arteries. Pairs of piezoelectric crystals were implanted within the subendocardium of the left anterior descending and the left circumflex coronary artery perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion.

Hof et al. (1990) used the Doppler method for measuring cardiac output in **conscious rabbits**.

Grohs et al. (1993) simultaneously assessed cardiac output with pulsed Doppler and electromagnetic flowmeters during cardiac stimulation.

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A.1.3.12

Hemodynamic Studies in Monkeys

PURPOSE AND RATIONALE

Prior to studies in human beings, studies of cardiovascular effects of new drugs in monkeys are necessary in some instances.

PROCEDURE

Rhesus monkeys of either sex, weighing between 5 and 8 kg are anesthetized with 20 mg/kg ketamine hydrochloride followed by 50 mg/kg pentobarbital-Na given slowly i.v. A small side-branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure recordings using a blood pressure transducer (P23 ID). Heart rate is determined from a conventional ECG lead by a biotachometer. Compounds are administered either intravenously or via a gastric fibroscope, e. g., Olympus XP 10, into the duodenum under visual control. The cardiovascular parameters are registered for a pretest period of 30 min and then during 60 min after intravenous administration or 2 h after intragastric administration of the test drug. Three to 6 animals are used for evaluation.

EVALUATION

Mean values \pm SD are calculated for the pretest period and for the cardiovascular effects every min for 5 min after i.v. administration and then every 5 min. After intragastric administration the values are registered every 5 min up to 30 min and then every 10 min. The values after administration of the test compound are compared statistically with the pretest values using the Student's *t*-test.

MODIFICATIONS OF THE METHOD

Lacour et al. (1993) studied cardiovascular parameters in conscious **cynomolgus monkeys** (*Macaca*

fascicularis). A silicone catheter (internal and external diameter 0.64 and 1.19 mm, respectively) was implanted under aseptic conditions into the thoracic aorta via a carotid artery after the monkeys had been anesthetized with 40 mg/kg ketamine and 0.5 mg/kg acepromazine intramuscularly. The vascular catheter (filled with an aqueous solution of 40% polyvinylpyrrolidone and 20% heparin) was inserted into a carotid artery. A patch of silicone was sewn around the artery to maintain the catheter in position, the latter being routed subcutaneously and exteriorized at the top of the head into a stainless steel connector. This connector was fixed to the skull with screws and dental cement, and sealed with a plug to protect the catheter from damage. The monkeys were permitted a 3-week minimum recovery period. Before the experiment was performed the monkeys were placed in a primate-restraining chair on several occasions, of gradually increasing duration, for experiment acclimatization.

Pulsatile arterial pressure was recorded by connecting the arterial catheter to a polygraph via a Statham P23Id pressure transducer. Mean arterial pressure and heart rate were derived from the pulse pressure signal and recorded. A catheter was inserted acutely into a saphenous vein for administration of compounds.

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A.1.3.13

Measurement of Cardiac Output and Regional Blood Flow with Microspheres

PURPOSE AND RATIONALE

The microsphere technique allows the measurement of cardiac output and regional blood flow. Using different radionuclides, repeated determinations are possible. The method is applicable not only for dogs, cats, and minipigs (Hof et al. 1980) but also for rats (McDevitt and Nies 1976; Bonnaccrossi et al. 1978; Ishise et al. 1980; Stanek et al. 1985) using microspheres of appropriate size.

PROCEDURE

Male Sprague-Dawley rats weighing 265–375 g are anesthetized with 35 mg/kg i.p. pentobarbital. The

right carotid and right femoral arteries are cannulated. Using pressure monitoring, a carotid cannula is manipulated into the left ventricle. Carbonized microspheres ($15 \pm 5 \mu$ diameter) labelled with ^{85}Sr are drawn into a glass injection chamber and suspended in 0.3 ml 6% dextran so that each chamber contains 60000 to 80,000 microspheres. The radioactivity in each chamber is determined by gamma scintillation counting before and after microsphere injection, the difference being the amount of radioactivity injected. The microspheres are injected into the left ventricle in a total volume of 0.8 ml 6% dextran over 20 s. Simultaneously, arterial blood from the femoral artery is withdrawn at 0.8 ml/min for 90 s with a syringe withdrawal pump.

EVALUATION

This reference blood sample is used to calculate the cardiac output by the formula:

$$\text{cardiac output} = \frac{\text{counts injected}}{\text{reference sample counts}} \times \frac{\text{reference sample withdrawal rate}}{\text{reference sample counts}}$$

After obtaining the reference sample, the animals are sacrificed with pentobarbital and the organs dissected, placed in counting vials, and counted for 5 min. Regional distribution of the cardiac output is calculated by comparing the radioactivity in each organ with the total injected radioactivity. Organ flow is determined by multiplying the cardiac output by the fractional distribution of the cardiac output to the organ.

CRITICAL ASSESSMENT OF THE METHOD

Problems associated with the microsphere technique in rats are the hemodynamic effects of the solutions used to inject the microspheres and the effects of blood withdrawal after repeated determinations (Stanek et al. 1985).

MODIFICATIONS OF THE METHOD

For repeated determinations, other nuclides have been used, such as ^{46}Sc , ^{51}Cr , ^{141}Ce , ^{125}I (Hof et al. 1980).

Kováč et al. (1992) used up to 5 radiolabelled microspheres (^{57}Co , ^{113}Sn , ^{85}Sr , ^{95}Nb and ^{46}Sc) for measurement of regional cerebral blood flow in cats.

Faraci and Heistad (1992) measured blood flow with radioactive microspheres (15μ diameter) labeled with ^{46}Sc , ^{95}Nb , ^{153}Gd , ^{85}Sr , and ^{141}Ce in anesthetized rabbits.

Grover et al. (1990), Gross et al. (1992) measured myocardial blood flow in dogs with the radioactive microsphere technique.

Kowallik et al. (1991) measured regional myocardial blood flow with multiple colored microspheres. The method yielded values very similar to those obtained with radioactive microspheres.

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A.1.3.14

Carotid Artery Loop Technique

PURPOSE AND RATIONALE

The carotid loop method, originally described by van Leersum (1911) for rabbits has been used by several authors (e. g., Child and Glenn 1938; Valli et al. 1967; O'Brien et al. 1971; Meyer et al. 1989) for measurement of blood pressure or blood sampling in conscious dogs and sheep (Lagutchik et al. 1992).

PROCEDURE

Male or female inbred Beagle or Labrador-HARRIER dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, continued with an infusion of 4–6 mg/kg/h. The animal is placed on a heated operating table. The skin on the ventral side of the neck is carefully shaved and disinfected. The course of the carotid artery is outlined by palpation along the tracheal border. About 2 cm of skin is taken on each side marking the width of the flap. The medial incision is made slightly above the thyroid cartilage and is extended caudal to a point about 1 cm lateral and 1 cm above the manubrium sterni. The lateral incision again lies about 2 cm from the line of the carotid artery and parallel to it. The lateral incision is only half as long as the medial one. The incisions are made down to the subcutaneous tissue over the platysma muscle. Between the skin and the muscle, the flap is undermined. All bleeding points are carefully clamped and tied.

The subcutaneous tissue, the platysma myoides muscle and the anterior fascia of the neck are incised in the course of the midline incision down to the plane of cleavage between the sternohyoid and sternomastoid muscle. By blunt dissection, these muscles are separated, disclosing at their depth the neurovascular bundle over which lies the internal jugular vein. The floor of the space so isolated is formed by the longus capitis muscle. By careful dissection, these muscles are separated at least 1 cm above and below the limits of the incision in the skin. The superior thyroid artery marks the uppermost portion of the carotid artery suitable for exteriorization. The plane of cleavage is followed caudal to the origin of the sternocleidomastoid muscle at the manubrium sterni. Throughout the limits of the incision the artery is dissected free from the internal jugular vein and then from the vagus nerve.

The first step in the exteriorization of the artery is the reapproximation of the muscle borders beneath the vessel by mattress sutures. In order to prevent tension on the completed loop due to contraction of the sternomastoid and sternohyoid muscle, it is important to reapproximate these muscles throughout their course. Sutures are placed at the edges of skin. The tubular flap of skin is then approximated loosely around the carotid artery. It is essential that the skin flap fits loosely around the artery. A continuous suture of fine silk is started at the place where the vessel emerges from the muscle borders. The suture is so placed as to include the artery in a sling of skin which isolates the vessel from the line of suture of the underside of the

completed loop. Finally, the proximal and distal quarters of the flap are closed with sutures, while the skin tube is closed with a continuous suture. Antibiotics are given locally and systemically.

One thickness of gauze is placed beneath the loop and along each border a strip of gauze in order to relieve the loop from the pressure caused by the remainder of dressings. Around the neck is wrapped a gauze bandage several turns of which have passed behind the forelimbs in order to prevent the dressing from riding upwards on the animal's neck. Over this is placed a plaster roll protecting the loop from the animal's efforts of scratching. The dressings are changed on the fifth and seventh day when the sutures can be removed.

Blood pressure measurements can be made according to Riva-Rocci's principle by placing an inflatable cuff around the loop.

CRITICAL ASSESSMENT OF THE METHOD

The carotid artery loop method needs some surgical experience and very meticulous caretaking of the animals.

MODIFICATIONS OF THE METHOD

Lewis et al. (1980) placed a CO₂ sensor using mass spectrometry and its through flow cuvette in a common carotid artery-to-jugular vein loop in anesthetized cats.

Meyer et al. (1989a, b) studied pulmonary gas exchange in panting dogs with an exteriorized carotid artery loop.

Kaczmarczyk et al. (1979) used conscious, chronically instrumented dogs with electric flow probes around the left renal artery and a carotid loop to study postprandial volume regulation.

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A.1.3.15

Measurement of Heart Dimensions in Anesthetized Dogs

PURPOSE AND RATIONALE

The measurement of the heart dimensions allows to localize the effect of a drug on the activity of the heart. An ultrasonic technique is used for continuous measurement of left ventricular dimensions. Compounds are tested with potential anti-anginal activity due to the reduction of left ventricular diameter. The test is used to evaluate the influence of drugs on left ventricular external and internal diameter in anesthetized dogs.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used for the test. The dog is anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium followed by subcutaneous injection of 2 mg/kg morphine. Respiration is maintained through a tracheal tube with N₂O/O₂ (3:1) using a positive pressure respirator.

Implantation of Ultrasonic Transducers

Ultrasonic transducers are constructed and implanted as described by Stinson et al. (1974).

To measure left ventricular external diameter (LVED), two ultrasonic transducers are fixed to the left ventricular wall. One crystal is sutured to the posterior wall within the rectangular area formed by the left circumflex coronary artery and the left posterior descending artery. The other one is placed near the first diagonal branch of the left anterior descending coronary artery. Exact positioning is assured with an oscilloscope.

To measure left ventricular internal diameter (LVID), the transducers are placed in the same anatomical area as for the epicardial crystals. However, they are pushed through the wall of the left ventricle through stab wound incisions. The crystals are positioned across the greatest transverse diameter of the left ventricle, one on the anterior and the other on the posterior endocardial wall.

Bleeding during the implantation procedure is controlled by umbilical tapes around the cranial and caudal veins and by purse string sutures at the implan-

tation sites. The pericardial incision and the chest is closed by sutures and the transducer wires are connected to the recording equipment.

In each dog, either LVED or LVID is measured together with the other hemodynamic parameters.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannulated femoral artery by a pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left A. carotis communis. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dp/dt_{max} is differentiated and heart rate is calculated.

Hemodynamic parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 30 min (control values), the test substance is administered by intravenous or intraduodenal injection.

Readings are taken at times 0, 15, 30, 45, 60, 75, 90 and 120 after drug administration. Left ventricular dimensions are measured at the end of the diastole and systole.

Characteristics:

- blood pressure
 - systolic blood pressure
 - diastolic blood pressure
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure
- left ventricular contractility, dp/dt
- heart rate, HR
- left ventricular external diameter, LVED
- left ventricular internal diameter, LVID

EVALUATION

Hemodynamic parameters, LVED and LVID [mm] are determined.

Changes in parameters after drug administration are compared to control values obtained during the 30 min pre-drug period.

Statistical significance is assessed by means of the paired *t*-test.

Since a change in the diameter of the left ventricle is a reasonable accurate index of left ventricular volume, a reduction of LVED or LVID with no change in dp/dt and HR can be considered as a strong indicator

for “venous pooling” and thus an anti-anginal activity of a compound.

Scores are allotted relative to the efficacy of standard compounds assessing the intensity as well as the duration of the effect.

Standard data:

| | | LVED [mm] | LVID [mm] |
|----------------------|-------------------|--------------|---------------|
| Nitroglycerin | 0.005 mg/kg, i.v. | -0.9 20 min | -1.2 30 min |
| Isosorbide-dinitrate | 0.1 mg/kg, i.v. | | -0.6 120 min |
| Molsindomine | 0.2 mg/kg, i.v. | -2.1 >60 min | -1.4 >120 min |
| Nifedipine | 0.1 mg/kg, i.v. | | +1.2 120 min |

MODIFICATIONS OF THE METHOD

Novosel et al. (1992) measured the dimensions of the right ventricle with microsonometry in anesthetized rabbits.

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A.1.3.16

Telemetric Monitoring of Cardiovascular Parameters in Rats

PURPOSE AND RATIONALE

Radiotelemetry allows the recording of cardiovascular parameters in conscious, free-moving animals. Several authors (Brockway et al. 1991; Mattes and Lemmer 1991; Guiol et al. 1992; Morimoto et al. 1992; Basil et al. 1993; Brockway and Hassler 1993; Lemmer et al. 1993, 1994, 1995; Calhoun et al. 1994; Diamant et al. 1993; Kramer et al. 1993a, 1995; Griffin et al. 1994;

Kuwahara et al. 1994; Sato et al. 1994, 1995; van den Buuse et al. 1994; Kinter 1996; Becker et al. 1997; Witte et al. 1998) used commercially available systems with some modifications to study the circadian rhythm of blood pressure and the influence of drugs on heart rate, blood pressure and motility in rats.

PROCEDURE

The telemetry and data acquisition system (e. g., Data Sciences International, Inc., St Paul MN) consists of four parts:

1. the implantable transmitter, which measures the pressure. This device contains a highly stable, ion-implant, semiconductor, strain-gauge sensor and battery-powered electronics to process the information from the pressure sensor and to telemeter it from within the animal. Arterial pressure is transmitted to the sensor via a 0.7-mm diameter, fluid-filled catheter;
2. the receiver which detects the signal from the implanted transmitter and converts it to a form readable by computer;
3. the pressure reference module, which measures atmospheric pressure to allow for the telemetered absolute pressure to be converted to a gauge pressure;
4. the data acquisition software, which accepts data from the reference module and the receivers, filters corrupt samples from the incoming data stream, converts the telemetered pressure to millimeters of mercury, subtracts atmospheric pressure from the telemetered pressure, and stores the data for retrieval, plotting, and analysis.

Under pentobarbital anesthesia, the telemetry transmitter is implanted into rats. The descending aorta is exposed between the renal arteries. A vascular clamp is made by putting two surgical threads on the proximal and distal part of the artery. The catheter tip is inserted through an incision in the vessel. A drop of cyanoacrylate glue is applied to the dried entry point. The transmitter is sutured to the abdominal musculature.

EVALUATION

Data from individual animals are recorded over long periods of time which allow the investigator to follow the circadian rhythm under several experimental conditions.

MODIFICATIONS OF THE METHOD

Hess et al. (1996) monitored pulmonary arterial pressure in freely moving rats by inserting the sensing

catheter of a telemetric system through a small hole and pushing it into the pulmonary artery.

Further cardiovascular studies in rats using the telemetric system were reported by Sgoifo et al. (1998), Webb et al. (1998).

Kramer et al. (1993b) used telemetry to record electrocardiogram and heart rate in freely moving mice.

Carlson and Wyss (2000) used small telemetry probes for long term recording of arterial pressure and heart rate in mice after implantation to the carotid artery or the abdominal aorta.

DePasquale et al. (1994) used radiotelemetry to monitor cardiovascular function in conscious guinea pigs.

Telemetric ECG recordings in **cardiomyopathic hamsters** were reported by Desjardins et al. (1996).

Van den Buuse and Malpas (1997) studied cardiovascular parameters in **rabbits** by radiotelemetry.

Astley et al. (1991), Smith et al. (1993) used telemetric systems to monitor cardiovascular responses in **baboons**.

Schnell and Wood (1993) measured blood pressure and heart rate by telemetry in conscious, unrestrained **marmosets**.

An ultrasonic blood flowmeter telemetry system for **cats** and rabbits has been described by Yonezawa et al. (1989, 1992).

Telemetry was used by Symons et al. (1992) to monitor the severity of events representing myocardial dysfunction in **miniswine**.

Savory and Kostal (1997) applied the telemetric system for chronic measurement of cardiovascular and other parameters in **chicken**.

Radiotelemetry has also been used for other pharmacological experiments, such as field potential analysis by radioelectroencephalography (see Sect. E.1.2.6), step through passive avoidance (see Sect. F.3.1.2), shock-prod burying test in rats (see Sect. E.2.5.3), measurement of body temperature (see Sect. H.4.0.2) and motility in rats and mice (Clement et al. 1989; Guillet et al. 1990; Diamant et al. 1993; van den Buuse 1994).

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A.1.3.17

Cardiovascular Effects

After Intracerebroventricular Administration

PURPOSE AND RATIONALE

Several drugs, like α_2 -adrenergic agonists, act primarily at central sites. Their effects can be most clearly demonstrated after injection into the cerebroventricular system. The first experiments have been performed in cats. The method has been adapted to rats.

PROCEDURE

Rats of either sex weighing 250–350 g are anesthetized with 100 mg/kg hexobarbital i.p. The scalp is cut in a sagittal line. With a dental drill a hole of 1–1.5 mm diameter is drilled through the cranial bone 1 mm lateral and 2 mm caudal of the bregma. A PVC-catheter is introduced perpendicular to the bone to a depth of 3 mm in order to reach the lateral cerebral ventricle. The catheter is fixed with dental cement and the wound closed. Test substances are administered through the catheter. To measure blood pressure one catheter is placed in one carotid artery and connected to a Statham transducer. Blood pressure and heart rate are recorded on a polygraph over a period of at least 30 min. For long acting drugs registration periods up to 2 h are necessary. After the experiment, the animal is sacrificed and the brain removed to confirm the site of injection.

EVALUATION

Systolic and diastolic blood pressure as well as heart rate after intracerebroventricular injection are expressed as percentage of pretreatment values. The response is compared with the standard clonidine which is effective in doses of 4–60 μ g.

MODIFICATIONS OF THE METHOD

Based on the work of Feldberg et al. (1954) and Hayden et al. (1966), Mastrianni et al. (1986) developed an

intracerebroventricular perfusion system for the study of centrally acting antihypertensive drugs in the rat. The antihypertensive effect of clonidine could be observed over several hours.

Methods used to detect central hypotensive activity of drugs have been reviewed by Timmermans (1984).

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A.1.3.18

Influence on Orthostatic Hypotension

PURPOSE AND RATIONALE

Orthostatic hypotension with dizziness up to unconsciousness is a syndrome occurring in many human individuals. Moreover, several drugs are known to cause orthostatic hypotension. In several animal species, such as rabbit, cat and dog, this syndrome can be evoked by changing the usual horizontal position into a vertical position with the head upwards using a tilting table.

PROCEDURE

Cats of either sex weighing 2.0–3.0 kg are temporarily anesthetized with ether. Anesthesia is maintained by intravenous injection of 70 mg/kg chloralose. The animal is fixed with its legs on a heated operating table which can be tilted by 90 degrees. The carotid artery is cannulated for measuring blood pressure through a Statham P 23 Db transducer on a 6 channel Helige recorder. The femoral vein is cannulated for injection of the test compound. After the blood pressure is stabilized for 30 min, the animal is quickly tilted to a vertical position for 1 minute. Due to the change of position and gravitational force, there is a rapid fall in blood pressure which recovers as soon as the animal is restored to its original position. After taking the control reading, the test compound is administered intravenously and the same procedure is repeated. The fall in blood pressure is recorded.

EVALUATION

A significant increase in postural hypotension with respect to the control would indicate that the test compound may produce orthostatic hypotension in human. Moreover, some compounds, like sympathomimetics, can reduce or prevent postural hypotension.

MODIFICATIONS OF THE METHOD

Sponer et al. (1981) described a method for evaluating postural hypotension in conscious **rabbits** placed on a tilting table whereby blood pressure was measured from the central artery of the ear.

Takata et al. (1999) reported a rabbit model for evaluation of chlorpromazine-induced orthostatic hypotension.

Humphrey and McCall (1982) described a model for predicting orthostatic hypotension during acute and chronic antihypertensive drug therapy in **rats** anesthetized with chloralose, urethane and pentobarbital using a heated tilting table.

Lee et al. (1982) evaluated postural hypotension induced by drugs in conscious restrained normotensive rats. Blood pressure was recorded after cannulation of the femoral artery under light ether anesthesia. A special tilting table was build for simultaneous studies in four rats.

Martel et al. (1996, 1998) studied the phenomenon of cardiovascular deconditioning observed in crew members of space flights in rats after tail suspension.

Socci et al. (2000) studied cardiovascular responses to simulated microgravity in Sprague-Dawley rats. Microgravity is known to induce orthostatic intolerance and baroreflex impairment in astronauts. The authors used 30° head-down tilt, 24-h whole-body suspension or 7-day tail suspension to mimic microgravity and to find treatment ameliorating the symptoms.

Baum et al. (1981) studied antihypertensive and orthostatic responses to drugs in conscious **dogs**. A catheter was placed in the subclavian artery for measurement of blood pressure and exteriorized at the back of the neck some days prior to the experiment. The animals were placed into a sling and tilted to the 90° upright position for periods of 60 s. every hour by lifting their forelimbs. Blood pressure response before and after treatment with test drugs was measured.

A none human **primate** model for evaluating the potential of antihypertensive drugs to cause orthostatic hypotension was described by Pals and Orley (1983) Polyvinyl catheters were implanted in the abdominal aorta and the vena cava via an external iliac artery and vein to **cynomolgus monkeys** during ketamine anesthesia. The catheters were routed subcutaneously from

the groin area to the top of the head and exteriorized. After recovery the animals were placed in restraining chairs allowing the change from vertical to horizontal position.

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A.1.3.19

Bezold-Jarisch Reflex

PURPOSE AND RATIONALE

The circulatory collapse after intravenous injection of veratrine has been first described in cats and is known as BEZOLD-JARISCH-reflex (Bezold and Hirt 1867; Jarisch and Richter 1939a, b, Jarisch 1940; Aviado and Guavera-Aviado 2001). Fleckenstein et al. (1950) recommended this as a suitable animal model of shock.

The original observation was a triphasic blood pressure response in cats or dogs characterized by a short lasting fall in blood pressure accompanied by bradycardia, followed by a short lasting increase and then a long-lasting decrease of blood pressure after intravenous injection of veratridin or other veratrum alkaloids.

Kalkman et al. (1984) showed that three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin observed in the Bezold-Jarisch reflex.

The Bezold-Jarisch reflex has been studied in several species, such as **cats** (Takei et al. 1995; Vayssettes-Couchay et al. 1997), **dogs** (Zucker and Cornish 1981; Barron and Bishop 1982; Harron and Koberinger 1984; Giles and Sander 1986; Baugh et al. 1989; Watson et al. 1995), **ferrets** (Andrews and Bhandari 1993), **rabbits** (Chen 1979), guinea pigs, **rats** (Fozard 1984; Gyls et al. 1988; Cohen et al. 1989; Blower 1990; Miyata et al. 1991; Turconi et al. 1991; Matsumoto et al. 1992; Meller et al. 1992; Robertson et al. 1992; Kishibayashi et al. 1993; Geissler et al. 1993; Haga et al. 1994; Hegde et al. 1994; Eglen et al. 1995; Göthert et al. 1995; Delagrangé et al. 1996; De Vries 1997; Malinowska et al. 2001; Godlewski et al. 2003) and **mice** (Eglen et al. 1994; Middlefell et al. 1996), whereby species differences have been observed (Yamamoto et al. 1995).

In cats and dogs, the Bezold-Jarisch reflex was elicited by veratrine and veratridine, but also by capsaicin and the 5-HT₃ receptor agonists 2-methyl-5-HT, phenylbiguanide, chlorophenylbiguanide and serotonin itself.

In rats, mostly 5-HT or 2-methyl-5-HT were used as stimuli to characterize 5-HT₃ receptor antagonists.

PROCEDURE

Male Sprague Dawley rats weighing 250–380 g are given food and water ad libitum, except those used for intraduodenal drug administration; these rats are deprived of food overnight. The animals are anesthetized by intraperitoneal injection of 1.5 g/kg urethane. Body temperature is maintained at 37°C by placing the animal on a heating pad. The left jugular vein or duodenum, trachea and left femoral vein are cannulated for drug administration (i.v. or i.d.), facilitation of respiration and injection of 2-methyl 5-HT, respectively. Heart rate is derived from a limb lead II ECG monitored via subdermal platinum electrodes and is recorded with amplifiers on a polygraph. A dose-response curve to 2-methyl 5-HT (5–100 µg/kg, i.v.) is constructed in each rat to establish a submaximal dose (usually 10 or 20 µg/kg, i.v.) which elicits a reproducible bradycardic response. Each rat receives then a single dose of test drug or standard and is then challenged with 2-methyl 5-HT at 5, 15, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min post dosing. A separate group of rats receiving vehicle (saline for i.v.,

deionized water for i.d.) is similarly tested in each study.

EVALUATION

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls. Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's multiple comparison test.

MODIFICATIONS OF THE METHOD

Harron and Kobinger (1984) used capsaicin to elicit the Bezold-Jarisch reflex in anesthetized artificially respired dogs pretreated with a beta-adrenoceptor antagonist to evaluate the activity of clonidine-like drugs on central α_2 adrenoceptors after intracisternal administration.

The Bezold-Jarisch reflex in rats has been used for evaluation of 5-HT₃ receptor agonists (Rault et al. 1996; López-Tudanca et al. 2003).

Rocha et al. (2003) found an enhancement of the Bezold-Jarisch reflex in the acute phase of myocardial infarction of the anesthetized rabbit.

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A.1.3.20

Endotoxin Induced Shock

PURPOSE AND RATIONALE

Many bacterial infections as well as allergic reactions are known to induce pathophysiological events that may lead to shock in man. When experimental animals are injected with endotoxin and galactosamine, shock and death occur in all untreated animals 5–7 h after injection. The endotoxin induced shock is marked by pulmonary embolism, bronchospasm and renal failure. Bacterial liposaccharides (endotoxins) play an important role in the pathogenicity of Gram-negative infections.

The reactivity of animals to endotoxin may be enhanced by simultaneous administration of galactosamine. Galactosamine is a specific hepatotoxic agent that leads to early metabolic alterations and consequent cellular liver damage. The following procedure is used to detect compounds that prevent the occurrence of endotoxin-induced shocks.

Cardiovascular parameters of endotoxin induced shock are greatly influenced by various anesthetics. For this reason, a model was proposed by Brackett et al. (1985) and Schaefer et al. (1987) to study the circulatory shock pattern after endotoxemia in conscious unrestrained rats.

PROCEDURE

Male Sprague-Dawley rats weighing 300 ± 10 g are anesthetized with 5% enflurane. A tracheal cannula is connected to a rodent respirator delivering 2% enflurane. Via the right jugular vein the tip of one catheter is placed just adjacent to the right atrium for injection of endotoxin, monitoring of central venous pressure, and rapid injection of room-temperature saline to produce thermodilution curves for calculation of cardiac output. The right carotid artery is cannulated with a thermistor-catheter combination for measurement of thermodilution cardiac output curves and aortic blood pressure. The thermistor tip is placed in the aortic arch just distal to the aortic valve. The catheters are guided under the skin exiting through the back of the neck just below the base of the skull.

The animals are allowed to regain consciousness and are then placed in cages that allow unrestrained movements about the cage at all times throughout the study with no further handling. The experimental animals receive a 20-sec infusion of 40 mg/kg endotoxin (*E. coli*, Difco) being paired with sham animals with identical catheters but receiving an equal volume of saline. Test compounds are injected intravenously

10 min prior to endotoxin injection. Cardiac outputs are measured using the thermodilution technique by rapidly injecting a volume calculated to deliver 100 μ l of room temperature saline to the circulatory system. Central venous and aortic blood pressure and heart rate are continuously monitored for the following 4 h. Cardiac output measurements are made 5, 15, 30, 60, 120, 180, and 240 min after endotoxin. At the end of the study, the animals are sacrificed and the catheters checked visually to ensure proper placement.

EVALUATION

Central venous pressure, arterial pressure, and cardiac output of drug treated animals receiving endotoxin are compared with animals receiving endotoxin only and saline sham treated animals. Furthermore, cardiac index, total peripheral resistance, and stroke volume are calculated. The small intestines of all rats are examined for severity of hemorrhage using a five point scale. Repeated-measures analysis of variance is used to analyze the data.

MODIFICATIONS OF THE METHOD

Lindenbaum et al. (1990) studied the effect of *E. coli* endotoxin on cardiovascular parameters of anesthetized **dogs**. Inhibition of the deterioration of metabolic functions and improvement of cardiovascular parameters were found after cocarboxylase treatment.

Endotoxin induced shock has been tested in **mice** (Galanos et al. 1979). Groups of 10 male C57BL/6 mice weighing 20–22 g are injected intravenously with a mixture of 0.01 μ g of *Salmonella abortus equi* lipopolysaccharide and 7.5–15 mg galactosamine in 0.02 ml phosphate buffered saline. The test compound is administered either intravenously at the same time or orally 45 min prior challenge. Twenty-four hours later, the number of surviving mice is determined.

Metz and Sheagren (1990) reviewed the effects of ibuprofen in animal models of septic shock.

Von Asmuth et al. (1990), Luongo et al. (1998), and Cuzzocrea et al. (2004) described a zymosan-induced shock model in **mice**. Male CD mice (20–22 g) were treated intraperitoneally with zymosan (500 mg/kg, suspended in saline solution) or with zymosan and drug (rosiglitazone 3 mg/kg, intraperitoneally) at 1 and 6 h after zymosan. Eighteen hours after administration of zymosan, animals were assessed for non-septic shock. Clinical severity of systemic toxicity was scored for the whole experimental period (12 days) in the mice after zymosan or saline injection on a subjective scale ranging from 0 to 3: 0 = absence, 1 = mild,

2 = moderate, 3 = serious. The ranging scale was used for each of the toxic signs (conjunctivitis, ruffled fur, diarrhea, and lethargy) observed in the animals. The final score was the sum of the single evaluation (maximum value 12).

Overbergh et al. (2003) studied acute shock induced by antigen in NOD mice. The 8-week-old NOD, BALB/c, and C57BL/6 mice were immunized by injection of 100 μ g antigen [Hen egg white lysozyme (HEL), GAD65 (p524–542) (SRLSKVAPVIKARMMEYGT), bovine insulin B (ins-B) chain, heat shock protein (hsp)-65, PLP peptide (amino acids 135–151), ovalbumin, keyhole limpet hemocyanine (KLH), and tetanus toxin] emulsified at a 1:1 concentration in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich., USA) in the hind footpads. NOD-*scid* mice were injected with 100 μ g HEL antigen suspended in CFA. All mice were reinjected with the same antigen 3 weeks later in a similar manner. Clinical evolution and survival rate after sensitization with various peptides were monitored in different mouse strains. Shock was characterized by pilo-erection, prostration, erythema of the tail, ears, and footpads, and dyspnea with shallow breathing. Serum for antibody measurement and spleens for quantification of mRNA levels were collected before immunization and again before booster administration.

Baldwin et al. (1991) tested the effect of Polymyxin B on experimental shock from meningococcal lipooligosaccharide and *Escherichia coli* lipopolysaccharide endotoxins in anesthetized **rabbits**.

Muacevic and Heuer (1992) tested the effect of platelet-activating factor antagonists in anesthetized rats.

Otterbein et al. (1993) tested the effects of peptides on survival of mice injected with 50 mg/kg lipopolysaccharide endotoxin in mice and on survival of rats with fecal peritonitis.

Mountz et al. (1995) reported an increased susceptibility of fas mutant **MRL-Ipr/Ipr mice** to Staphylococcal enterotoxin B-induced septic shock.

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A.1.3.21

Hemorrhagic Shock

PURPOSE AND RATIONALE

Hemorrhagic shock is one of the most severe consequences of accidents. Several animal models in various species have been developed to resemble the conditions in man and to test therapeutic or prophylactic measures (Lamson and de Turk 1945; Selkurt and Rothe 1961; Mills 1976). A method for hemorrhagic shock in anesthetized as well as in unanesthetized rats has been described by van der Meer et al. (1987). Experimental hemorrhagic shock is defined as a situation in which the cardiovascular system, after a period of hypovolemia followed by complete re-infusion of the shed blood, gradually deteriorates ending in the death of the animal.

PROCEDURE

Female rats weighing 170–190 g are anesthetized by i.p. injection of sodium pentobarbital, 25 mg/kg, followed after 20 min by 20 mg/kg, and kept in a chamber at 30°C and relative humidity over 80%. The left femoral vein is cannulated for application of the test drug. The right common iliac artery is cannulated and the cannula (polyvinyl chloride, 14 cm long, inner diameter 2 mm) is filled with heparin and exteriorized in the neck. After intraarterial injection of 0.2 ml heparin 500 IU/ml, the cannula is connected to a siliconized calibrated glass reservoir (inner diameter 18 mm), the height of which can be changed to adjust the surface of the shed blood to a fixed level.

The test drug is injected i.v. 5 min prior to bleeding. Bleeding is performed against (at heart level) 30 mm Hg for 1 h, 25 mm Hg for 0.5 h, 30 mm Hg for 1 h, 25 mm Hg for 0.5 h, and finally 30 mm Hg for 1 h. The shed blood is partially taken up again spontaneously. After 4 h, re-infusion is started by increasing the pressure to 60 mm Hg for 5 min, to 80 mm Hg for 5 min, and (if necessary) to 100 mm Hg. During the hypovolemic phase respiration becomes gradually slower. If respiration arrest is imminent 0.5 ml 5% glucose are injected intra-arterially, thus avoiding death during the period of hypovolemia. Practically all rats die at an average of 4 h after complete re-infusion.

EVALUATION

Survival time is taken as the time between complete re-infusion and death. Average survival time of treated animals is compared with that of controls. Furthermore, after autopsy the number of gastrointestinal lesions, subendocardial hemorrhage, kidney tubular necrosis and liver cell necrosis are registered by histological examination.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that hemorrhagic shock does not reflect the situation of traumatic shock in man in every aspect, the condition is close enough to use the model for testing compounds which potentially inhibit or ameliorate shock in man.

MODIFICATIONS OF THE METHOD

A method to study hemorrhagic shock in dogs has been described in detail by Mills (1967). Large dogs weighing 20–30 kg are anesthetized by an i.v. injection of 25 mg/kg sodium pentobarbital. The animals are respirated by means of a Harvard respirator set at a stroke volume of 400 ml and a rate of 20 respiration/min. Blood pH is regulated between 7.37 and 7.42

by varying the gas flow between 100% O₂ and a mixture of 95% O₂ and 5% CO₂. Central arterial blood pressure is recorded by inserting a catheter through one femoral artery to the aortic arch. Pulmonary artery pressure is measured by inserting a PE 50 catheter through a small neck vein, reaching the right ventricle and allowing to float into the pulmonary artery. The right atrial catheter is also inserted through a small neck vein. After the chest is opened, the left atrial catheter is tied in place through a small opening in the left atrial appendage. Blood flow is measured in the ascending aorta (cardiac output), carotid, superior mesenteric, renal and femoral arteries using electromagnetic flowmeters. Furthermore, pulse rate is monitored from the electrocardiogram.

The test drug is injected i.v. 10 min prior bleeding. Blood is removed either at a specific volume or until a selected reduction of blood pressure has occurred. The cardiovascular parameters of treated animals are compared with those of controls.

Shock associated with hemoconcentration was produced in dogs by Davis (1941) by bleeding from the carotid artery and injections of 25% sodium chloride solution subcutaneously in doses of 25 ml.

The effect of insulin on glucose uptake in the soleus muscle of rats during hemorrhagic shock was studied by Chaudry et al. (1975).

Bauer et al. (1995) used hemorrhagic shock in rats to evaluate the influence of interleukin-1 on leukocyte-endothelial cell interactions and the microcirculation in the liver by means of intravital microscopy after application of an interleukin-1 receptor antagonist.

Kitajima et al. (1995) studied gastric mucosal injury induced by hemorrhagic shock in rats.

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A.1.3.22

Tourniquet Shock

PURPOSE AND RATIONALE

Compression of extremities in man by heavy objects for periods of several hours results in the so-called crush syndrome. The rescued individual shows immediately a favorable response to therapy, but within a few hours symptoms of shock develop followed by signs of progressive renal damage leading to death (Duncan and Blalock 1942). Moreover, arterial bleeding after accidents needs the applications of tourniquets. During surgical procedures on extremities a tourniquet may be necessary (Wilgis 1971), the time of which has to be limited in order to avoid fatal consequences. The pathophysiological mechanisms of tourniquet induced shock remain still to be elucidated. Nevertheless, animal models in rats (Chandra and Dave 1970), rabbits (Little 1974), and dogs (Goto et al. 1988) had to be developed to evaluate drugs capable to inhibit the fatal consequences of crush and tourniquet shock.

PROCEDURE

Wistar rats of either sex weighing 250–280 g are anesthetized with phenobarbital. The tourniquets consist of rubber tubes (internal diameter 4 mm, external diameter 5.8 mm). Both tights are fastened by the rubber tubes and the pressure which is monitored by a miniature pressure sensor and an amplifier (e. g. Kyowa Electronic Instruments Co, Tokyo) is adjusted to 1.5 kg/cm². The rubber tubes are knotted and the sensor removed. After 3 h the animals are treated with the test compound or the control solution. The tourniquet is left in place for 6 h while the animals remain under pentobarbital anesthesia. Then, the rubber tubes are removed, and the rats are returned to their cages. Within a few min, the reperfused hind limbs, which have been pale blue, turn pink. The animals are then allowed free access to food and water. Blood is withdrawn at different intervals during the tourniquet and afterwards for measurement of hematocrit, transaminases, urea nitrogen and total protein. Time to death is registered.

EVALUATION

Statistical evaluation of the survival intervals is performed with the log rank test according to Peto et al. (1976). Blood chemical data are analyzed using the Kruskal-Wallis (1952) rank sum test. Multiple comparisons are corrected by the Bonferroni's method (1980).

CRITICAL EVALUATION OF THE METHOD

These methods are valuable to find drugs effective in this life-threatening situation.

MODIFICATIONS OF THE METHOD

Ghussen et al. (1979) studied the effect of methylprednisolone on the experimental tourniquet shock in **dogs**.

Haugan and Kirkebo (1984) used a model in anesthetized **rats** with tourniquet shock by bilateral hind-limb occlusion for 3 1/2 h, and burn shock by scalding the hind 50% of the body surface for 30 s in 90°C water.

Horl and Horl (1985) investigated the effect of tourniquet ischemia on carbohydrate metabolism in **dog** skeletal muscle

Sáez et al. (1982) followed the time course of appearance of lactic dehydrogenase enzymes in the serum of **rats** after different periods of ischemia by bilateral application of rubber band tourniquets to the hind legs.

Sáez et al. (1986) studied the effects of allopurinol on biochemical changes of the gastrocnemius muscle in rats subjected to tourniquet shock followed by reperfusion.

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A.1.3.23**Heat Stroke****PURPOSE AND RATIONALE**

Heat stroke is a medical emergency where quick diagnosis and treatment of victims are essential for positive prognosis. Several animal models have been established by investigators in heat related studies. Rats (Francesconi and Mager 1978; Hubbard et al. 1977, 1979; Kielblock et al. 1982), rabbits (Shih et al. 1984), dogs (Bynum et al. 1977) and sheep (Tayeb and Marzouki 1990) are considered to be the most suitable models because of their similarity to man in response to high temperature.

PROCEDURE

Male Sprague Dawley rats weighing 450 to 550 g are fasted 18–24 h before the experiment. For prevention studies the animals are treated subcutaneously 1 h before either being restrained in an appropriate wire cage which is placed into an environmental chamber set at 41.5°C ambient temperature or being exercised in a motor-driven treadmill. Core temperature (rectal probe inserted 6.5 cm) are measured using copper/constantan thermocouples in conjunction with a thermocouple reference oven and a 10-channel data acquisition system with a teletype printout. After

reaching exhaustion or a predetermined core temperature, all rats are monitored at 26°C ambient temperature while resting in plastic cages lined with wood shavings. After recovery, animals are returned to their cages and allowed water but no food for 24 h.

EVALUATION

LD_{50} values are determined in treated and control animals.

MODIFICATIONS OF THE METHOD

Kielblock et al. (1982) analyzed cardiovascular function by direct recording of arterial blood pressure and ECG-analysis.

Francesconi and Mager (1978) studied pathochemical indices, such as serum lactate concentration, potassium levels and plasma creatine phosphokinase.

Kregel et al. (1988) investigated peripheral vascular responses to hyperthermia in the rat by implantation of Doppler flow probes on the superior mesenteric, left iliac or left renal, and external caudal arteries. They concluded that a selective loss of compensatory vasoconstriction triggers the cascade of events that characterize heat stroke.

Shido and Nagasaka (1990) studied thermoregulatory responses to acute body heating in rats acclimated to continuous heat exposure. Indirect external warming was performed by raising the jacket water temperature surrounding the calorimeter from 24 to 39°C. Intraperitoneal heating was made through an electric heater implanted chronically in the peritoneal cavity.

Chiu et al. (1995) reported an increased survival in rat heatstroke by reducing hypothalamic serotonin release after administration of interleukin-1 receptor antagonist.

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A.1.3.24

α - and β -Adrenoreceptors in the Mouse Iris

PURPOSE AND RATIONALE

A simple method to test mydriatic substances is the test on the mouse pupil as described by Pulewka (1932). The diameter of the pupil is narrowed by intensive light illumination. A dose-dependent increase of pupil diameter can be achieved by intraperitoneal application of atropine and synthetic mydriatics (Ing et al. 1950; Burn et al. 1950). The mydriatic effect of hexamethonium analogues has been measured by Blackman et al. (1956). Mydriasis is induced by norepinephrine, epinephrine and isoproterenol and can be antagonized by α - or β -blockers (Freundt 1965).

PROCEDURE

Male mice weighing 15–20 g are used. They are kept for at least 30 min in separate beakers under bright illumination before the pupil diameters are measured with a dissecting microscope containing an arbitrary scale in the eyepiece. To make the illumination as uniform as possible, the beakers containing the mice are placed beneath long low-power fluorescent tubes and on top of glossy white paper. The pupil diameter is measured in mm before and at various time intervals after treatment. Groups of 5–10 mice are used for each dose of compound and for vehicle control.

To test sympatholytic activity, various doses of the α - or β -blocker are injected subcutaneously 30 min prior to intravenous injection of 0.1 mg/kg norepinephrine, or 0.05 mg/kg epinephrine, or 20 mg/kg isoproterenol. The effect of norepinephrine is blocked by α -blockers, but not by β -blockers, the effect of epinephrine by both α - and β -blockers, and the effect of isoproterenol by β -blockers, but not by α -blockers.

EVALUATION

The mean values of diameters in the groups treated with α - or β -blockers are compared with those of an-

imals treated with norepinephrine, epinephrine or isoproterenol only.

MODIFICATIONS OF THE METHOD

Edge (1953) used mydriasis in the mouse as a quantitative method of estimating parasympathetic ganglion block.

Håkanson et al. (1987) used the isolated iris sphincter of pigmented rabbits to test multiple tachykinin pools in sensory nerve fibres. The eyes were taken out within 1 min after sacrifice and opened by an incision 2–3 mm posterior to the limbus, followed by excision of the iris from the ciliary margin. The iris sphincter muscle was then opened, cut in half and mounted vertically on a Perspex holder in a 7 ml tissue bath maintained at 35°C. The mechanical activity after electrical stimulation was recorded isometrically using a force displacement transducer and a polygraph.

Kern (1970) used isolated sphincter and dilator muscles from human eyes obtained at autopsy for studies on sympathomimetics and adrenergic blocking agents. Cholinotropic and α - and β -adrenergic receptors were identified.

Responses to bradykinin and or capsaicin of the isolated iris sphincter were considered to be mediated by substance P released from the trigeminal nerve (Ueda et al. 1984).

Pupillary dilatation can be used as an index for central nervous system α_2 -adrenoceptor activation (Koss 1986).

Clonidine induces mydriasis which is mediated by α_2 -adrenoceptors located in the brain (Berridge et al. 1983; Hey et al. 1985). Blockade of presynaptically located α_2 -adrenoceptors is considered as a possible mechanism for antidepressant drugs. Mianserin was able to antagonize clonidine-induced mydriasis in the rat.

Gower et al (1988) studied a large number of psychotropic drugs in this model with the aim to reveal *in vivo* α_2 -adrenoceptor blocking effects of new compounds.

Male Wistar rats weighing 230–300 g were anesthetized with pentobarbital, 60 mg/kg i.p., and a polyethylene catheter was inserted into the femoral vein for drug administration. The rat's head rested on the base platform of a binocular Olympus microscope positioned so that the pupil diameter of the right eye could be measured by means of a micrometer inserted into one eyepiece of the microscope. A constant light intensity was maintained throughout the experiment. Rats were first injected with saline 25 min after anesthesia induction. The pupil diameter was measured

1 min after injection. Five min after measurement, mydriasis was induced by clonidine (0.1 mg/kg, i.v.) and the diameter was measured 1 min after injection. This was followed by the test compound, injected at 6 min intervals at increasing doses. The pupil was measured at 1 min after each injection. The dose inhibiting 50% of the clonidine-induced mydriasis (ID_{50}) was determined per rat from the cumulative dose-response curve.

Savontaus et al. (1997) studied the effect of an imidazoline derivative against detomidine-induced mydriasis in anesthetized rats.

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A.1.3.25 **α_2 -Adrenoceptor Blockade Measured *In Vivo* by Clonidine-Induced Sleep in Chicks****PURPOSE AND RATIONALE**

In young chicks, clonidine causes a loss of righting reflex which is antagonized by mianserin (Pommier et al. 1982). This phenomenon was used to measure α_2 -adrenoceptor blockade *in vivo* by Gower et al. (1988).

PROCEDURE

Male white Leghorn chicks are used either a few hours after hatching or 1 or 2 days later. Clonidine-induced loss of righting reflex (sleep) is determined with 8 animals at a time. Two animals are treated with placebo and 2 with each of 3 dose levels of the test compound. Tests with groups of 8 animals are continued until 10 animals are tested per dose level or placebo treatment. The chicks are marked with ink and injected intraperitoneally with placebo or the test compound. Ten min later, 1.2 mg/kg clonidine is injected into a leg muscle and the animals are placed individually in small Macrolon cages. The beginning of sleep time is defined as the moment at which the animals can be placed on their back and remain in this position. Sleep time is recorded until they return to their feet spontaneously or another attempt to put them on their back fails. Sleep time is recorded for a maximum period of 30 min.

EVALUATION

Statistical evaluations of differences in median sleeping times are done with the Mann-Whitney *U*-test. Dose-response relations for various drugs can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

Compounds with known α_2 -adrenoceptor blocking activity antagonize clonidine-induced sleep in chicks dose-dependently. Yohimbine is one of the most active compounds. However, also other centrally active compounds of which their main effect is not α_2 -blockade, reduce clonidine-induced sleeping time. One of the most potent is apomorphine acting on dopamine D₂-receptors. Therefore, the clonidine-induced sleeping test in chicks can not be regarded as highly specific for α_2 -adrenoceptors.

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A.1.3.26**Activity at β_1 - and β_2 -Adrenoceptors in the Rat****PURPOSE AND RATIONALE**

The relative potency of catecholamines as stimulants of β -adrenoceptor mediated responses vary in different tissues indicating the existence of two subtypes of β -receptors (β_1 and β_2) (Lands et al. 1967). β -adrenoceptors in the heart have been classified as being of the β_1 -subtype. β -adrenoceptors in the uterus, diaphragm, bronchioles and small intestine have been classified as being of the β_2 -subtype, since in these tissues, epinephrine is more potent than norepinephrine. These observations led to the development of selective agonists and antagonists. Isolated organs (see below) having predominantly one receptor subtype, such as the isolated heart and the isolated atrium for β_1 , and the isolated uterus or the isolated tracheal chain for β_2 , are used to test compounds for selective activity. Assessing both activities in the same animal *in vivo* results in the advantage that pharmacokinetic and metabolic influences of the drug being tested are the same for both parameters.

PROCEDURE

Female Sprague-Dawley rats (200–220 g) are anesthetized with 60 mg/kg pentobarbital i.p. prior to pithing (Gillespie and Muir 1967). The animals are artificially respired with room air using a Harvard small animal ventilator (90 strokes/min at a pressure of 7 cm H₂O). Body temperature is maintained by placing the animals on a heated operating table. The left carotid artery is cannulated for continuous monitoring of blood pressure via a Statham p23Id pressure transducer. The blood pressure signal is used to trigger an instantaneous rate meter for continuous monitoring of heart rate. A femoral vein is cannulated for intravenous administration of drugs.

A midline incision is made to expose one horn of the uterus. The ovarian artery is cut, tied and one horn dissected free from the ovary leaving the myometrial blood supply intact. A cotton thread is attached to the free end of the uterine horn, passed through a glass-jacketed organ bath and connected to an isometric (Pioden UF1) transducer for measurement of spontaneous contractions. A cannula is inserted into the peritoneal

cavity for administration of drugs by the i.p. route. The organ bath is positioned such that it surrounds the uterine horn without touching it. The tissue is perfused with Krebs-Henseleit solution being gassed with 95% O₂/5% CO₂ and maintained at 37°C. A resting tension of 0.2 g is applied to the tissue, which is allowed to stabilize until spontaneous contractions are constant over a period of 5–10 min. All recordings are made on a polygraph.

EVALUATION OF AGONISTS

Dose-response curves after i.v. injection are established for isoprenaline (nonselective between β_1 - and β_2 -adrenoreceptors), salbutamol (selective for β_2 -adrenoreceptors), and noradrenaline (selective for β_1 -adrenoreceptors) in increasing heart rate (beats/min) and decreasing the height of uterine contraction (calculated as percentage of the original amplitude). Animals given noradrenaline are pretreated with phenoxybenzamine (3.3 mmol/kg i.v.) in order to antagonize irreversibly the α -adrenoreceptors. Agonist dose-response curves ($n > 4$) on heart rate and uterine relaxation are carried out by assessing the activity of at least 3 doses of each agonist. New synthetic compounds can be tested after intraperitoneal administration additionally.

EVALUATION OF ANTAGONISTS

The ability of a non-selective β -blocker, such as propranolol (1 mmol/kg i.v.), a β_1 -selective β -blocker, such as atenolol, and a β_2 -selective β -blocker to inhibit responses to isoprenaline on both heart rate and uterine relaxation is assessed by comparing the log linear portion of the dose-response curve to isoprenaline in the absence and in the presence of the β -adrenoreceptor antagonist in the same animal. Dose ratios for each antagonist are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method described by Piercy (1988) has the advantage to measure both agonistic and antagonistic activity and to differentiate between effects on β_1 - and β_2 -adrenoreceptors. Compared to tests in isolated organs, *in vivo* activity can be determined after intraperitoneal or intraduodenal administration.

MODIFICATIONS OF THE METHOD

Härtfelder et al. (1958) studied the influence of various agents on the contractions of electrically stimulated **isolated uteri of rabbits and guinea pigs**.

Nathason (1985) evaluated the activity of β -blockers to inhibit the cardio-acceleratory effect of

systemically administered isoproterenol in **unanes-
thetized, restrained albino rabbits** together with the effect on membrane bound adenylate cyclase in homogenized ciliary process villi in order to find compounds selectively lowering intraocular pressure.

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A.1.3.27

β_1 - and β_2 -Sympatholytic Activity in Dogs

PURPOSE AND RATIONALE

Intravenous administration of isoprenaline (isoproterenol) stimulates β_1 -receptors of the heart which can be detected as an increase in contractility (dp/dt max). Intraarterial injection of isoprenaline stimulates β_2 -receptors of peripheral blood vessels leading to an increased peripheral blood flow. Therefore, a β_1 - or β_2 -blocking activity of a compound is revealed by the inhibition of the effects of isoprenaline. The following tests are used to evaluate β -blocking activity of drugs. A β -blocker screening is done in anesthetized dogs (a); in addition, the test allows a differentiation between β_1 - and β_2 -receptor activity and the determination of ED_{50} values (b).

PROCEDURE

Male or female Beagle dogs weighing about 20 kg are used. Animals are premedicated with 1 g Inactin(i.v.) and anesthetized by intravenous administration of 20 mg/kg chloralose and 250 mg/kg urethane. In addition, they receive a subcutaneous injection of 2 mg/kg morphine 1 h after the start of anesthesia. Animals are

heparinized. Respiration is maintained through a tracheal tube using a positive pressure respirator. End-expiratory CO₂ content is measured continuously; respiratory rate and depth of respiration are adjusted to 4.5–6 vol% end-expiratory CO₂. For administration of isoprenaline, a peripheral vein is cannulated.

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, a cannula inserted into a femoral artery is connected to a pressure transducer (Statham p 23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted via the left arteria carotis communis. LVEDP is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted. Peripheral blood flow in the femoral artery is measured with an electromagnetic flow probe.

Screening for β -Blocking Effects in Anesthetized Dogs

Following a steady-state period of 30–60 min, isoprenaline is administered intravenously 2–3 times to the anesthetized animal and hemodynamic parameters are recorded (control values = 100%). Then, the test substance is injected intravenously at cumulative doses (final concentrations of 0.01, 0.05 and 0.15 mg/kg). For each dose, 10 min “drug effects” are monitored by measuring hemodynamic parameters. Then the effect of isoprenaline is tested again (3 times).

In other experiments, a single dose of the drug is administered to determine the duration of action.

If a test compound does not show an inhibitory influence on isoprenaline effects, a second test compound is administered.

All hemodynamic parameters are registered continuously during the whole experiment.

Testing for β_1 - and β_2 -Blocking Effects; Determination of ED_{50}

Following a steady-state period of 30–60 min, isoprenaline is administered for i.v. administration (β_1 -test) twice at a dose of 0.5 μ g/kg and for intraarterial administration (β_2 -test) twice at a dose of 0.05 μ g/kg. Hemodynamic parameters are recorded (control values = 100%). Then, the test substance is injected intravenously at cumulative doses. Consecutively increasing doses are given at 15-min intervals. For each dose, 10 min “drug effects” are monitored by measuring hemodynamic parameters. Thereafter isoprenaline is given intravenously and 5 min later intra-arterially.

All hemodynamic parameters are registered continuously during the whole experiment.

Characteristics:

- blood pressure
 - systolic, BPs
 - diastolic, BPD
- heart rate
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- dp/dt max
- peripheral flow, A. femoralis
- ECG, lead II

Standard compounds:

- propranolol HCl
- practolol
- metoprolol tartrate

EVALUATION

β_1 -receptor antagonism is measured as a decrease in contractility (dp/dt max).

Inhibition of the isoprenaline-induced elevation of heart rate is considered as an indicator for non-selective β -blockade. For cardioselective β -receptor blockers the increase in dp/dt max is inhibited with lower doses of test drug than the rise in heart rate.

β_2 -receptor blockade by a test drug is measured as inhibition of the isoprenaline-induced increase in peripheral blood flow.

The different hemodynamic parameters are determined.

Percent inhibition of the isoprenaline-induced effects by a test compound is calculated and compared to the isoprenaline effects before drug administration (= 100%).

ED_{50} values for β_1 - and β_2 -antagonism are calculated by log-probit analyses. ED_{50} is defined as the dose of drug leading to a 50% inhibition of the isoprenaline effects.

An $ED_{50} \beta_1/ED_{50} \beta_2$ -ratio of < 1 indicates that a β -blocking agent predominantly influences β_1 -receptors (cardioselectivity).

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A.1.3.28 Intrinsic β -Sympathomimetic Activity in Reserpine-Pretreated Dogs

PURPOSE AND RATIONALE

β -blocking agents can be classified as

- β -blocking agents with intrinsic sympathomimetic activity (ISA),
- β -blocking agents with membrane stabilizing activity (MSA),
- β -blocking agents with organ selectivity (high affinity to heart β_1 -receptors).

In the following procedure with reserpine-pretreated dogs, β -blocking agents with intrinsic sympathomimetic activity can be identified. Reserpine administration 24 h before the start of the experiment leads to a depletion of catecholamine depots. Thus, it is possible to differentiate between indirectly acting sympathomimetics such as tyramine and directly acting ones such as noradrenaline.

This test is used to identify β -blocking drugs with intrinsic sympathomimetic activity.

PROCEDURE

Male or female Beagle dogs weighing about 15 kg are used. Twenty-four h before the test, dogs receive an intramuscular injection of 0.3 mg/kg reserpine. At the day of the experiment, the animals are anesthetized by intravenous administration of 10–20 mg/kg pentobarbital sodium. Respiration through a tracheal tube using a positive pressure respirator is controlled by measuring end-expiratory CO₂ concentrations (4–5 vol%).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, a femoral artery is cannulated and connected to a pressure transducer (Statham p 23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted into the left ventricle via the left common carotid artery. LVEDP is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted.

Experimental Course

The test substance is administered by continuous intravenous infusion of 0.02 mg/kg (1 ml/min) until a cumulative dose of 3 mg/kg is achieved (within approximately 150 min). Thereafter, the velocity of infusion is doubled (0.04 mg/kg, 2 ml/min). The test is finished when a cumulative dose of 7 mg/kg is achieved (after a total time of approximately 250 min).

Hemodynamic parameters are registered continuously during the entire experiment.

Characteristics:

- blood pressure
 - systolic blood pressure
 - diastolic blood pressure

- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVED
- dp/dt max
- heart rate, HR

EVALUATION

The different hemodynamic parameters are determined. As a measure for intrinsic sympathomimetic activity (ISA), the increase in dp/dt max and in heart rate are evaluated. Absolute and relative differences of these parameters in drug-treated animals are compared to vehicle control values.

Statistical evaluations are performed by means of the Student's *t*-test if $n > 4$.

Scores are allotted relative to the efficacy of standard compounds for intensity as well as for duration of the effect.

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A.1.3.29

Cat Nictitating Membrane Preparation (Ganglion Blocking Activity)

PURPOSE AND RATIONALE

Nicotinic acetylcholine receptors are involved in the ganglionic neurotransmission. Various subtypes are described for nicotinic acetylcholine receptors (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

The nictitating membrane of the cat has been used extensively in pharmacological studies to evaluate ganglion blocking activity because of the ease with which its movements can be recorded, because of the simplicity of its innervation (the purely adrenergic fibres have their cell bodies in the easily accessible superior cervical ganglion of the same site) and because its blood supply (via the external carotid artery) is accessible for intraarterial injections. Preganglionic and postganglionic stimulation allow the interpretation of the mode of action of vasoactive drugs.

PROCEDURE

The animal is anesthetized with 35 mg/kg pentobarbital sodium i.p. Tracheostomy is performed and a tra-

cheal cannula is inserted. On one side, the sympathetic nerve is exposed, separated from the vagus nerve and prepared in order to place electrodes for preganglionic and postganglionic stimulation. Preferably, the vagus nerve at this site is severed at the central end. The head of the animal is fixed in a head holder to prevent head movements. A linear transducer is fixed at the mid of the border of the nictitating membrane allowing the registration of the contractions on a polygraph. Preganglionic and postganglionic stimuli are exerted by a square wave stimulator, with a pulse width of 0.3 to 0.5 ms, an amplitude of 1–3 V, and a frequency of 20/min. The amplitude and pulse width varies from animal to animal. The sympathetic nerve is stimulated before and after the administration of the compound and the changes in the contraction of the nictitating membrane are noted. Furthermore, the response of the nictitating membrane to exogenous adrenaline is registered.

EVALUATION

The decrease of the response after drug application is expressed as percentage of the control before drug. Ganglionic blockers decrease the response to preganglionic stimulation but have no influence on postganglionic stimulation or exogenous adrenaline. Neuronal blockers decrease the response to both preganglionic and postganglionic stimulation but do not affect the response to exogenous adrenaline which may even be enhanced. α -receptor blockers decrease the response to both preganglionic and postganglionic stimulation as well as decrease the effect of exogenous adrenaline. Catecholamine uptake inhibitors increase the response to both preganglionic and postganglionic stimulation as well as enhance the response to exogenous adrenaline.

CRITICAL ASSESSMENT OF THE METHOD

The nictitating membrane preparation has been widely used for differentiation of cardiovascular effects. Since the use of higher animals such as cats has been limited to a great extent, this model is now being used only exceptionally.

As alternative, the contraction of the inferior eyelid of anesthetized rats after preganglionic electrical stimulation of the superior cervical ganglion has been recommended (Gertner 1956; Steinbrecher and Schmid-Wand 1986). In the modification used by Steinbrecher and Schmid-Wand (1986) the method is suitable for testing compounds with potential adrenergic and antiadrenergic activity but not for testing ganglion blocking activities.

Male Sprague Dawley rats are anesthetized with 100 mg/kg thiobutabarbital i.p. and kept on a heated operation table at a rectal temperature of 37°C. One femoral vein is cannulated and filled with 4% heparin solution. One femoral artery is cannulated for registration of blood pressure. Tracheotomy is performed and a polyethylene catheter of 5 cm length inserted. The head of the animal is fixed carefully. The vibrissae at the lower eyelid on the right side are cut, a thread attached at the margin of this eyelid and attached to a strain-gauge. To immobilize the musculature of the face, the mouth of the animal is sutured and the head support attached. The right sympathetic nerve is exposed, separated from the vagus nerve and prepared in order to place electrodes for preganglionic stimulation. For calibration, stimulation is performed twice with an interval until contraction is back to baseline. Furthermore, a dose of 0.001 mg/kg adrenaline is given as bolus injection. Eyelid contraction and blood pressure increase are recorded. Then the putative adrenergic blocker or the standard 1.0 mg/mg phentolamine are injected intravenously. Eyelid contraction after electrical stimulation or after adrenaline is reduced dose-dependently.

MODIFICATIONS OF THE METHOD

Quilliam and Shand (1964) assessed the selectivity of drugs by comparing the effects on ganglionic transmission and on the pre- and post-ganglionic nerves in the isolated superior cervical ganglion preparation of the rat.

Langer and Trendelenburg (1969) performed experiments with normal nictitating membranes of pithed cats as well as with isolated normal nictitating membranes.

Koss and Hey (1992) used frequency-dependent nictitating membrane responses by sympathetic nerve stimulation in anesthetized cats to determine the potential role of prejunctional histamine H₃ receptors.

Gurtu et al. (1992) used contractions of the cat nictitating membrane to explore the effects of calcium channel blockers on neurotransmission *in vivo*, by comparing the effects of verapamil and nifedipine on contractions of nictitating membrane following either electrical stimulation of the superior cervical ganglion or intravenous injection of phenylephrine.

Koss (1992) compared the peripheral and central nervous system sympatholytic actions of prazosin using the cat nictitating membrane. Submaximal contractions of the nictitating membranes were evoked by electrical stimulation of the preganglionic cervical

sympathetic nerve trunk and by stimulation of the posterior hypothalamus in anesthetized cats.

Badio et al. (1996) evaluated spiropyrrrolizidines, a new structural class of blockers of nicotinic receptor channels with selectivity for ganglionic type receptors in rat pheochromocytoma PC12 cells (with an $\alpha_3\beta_{4(5)}$ -nicotinic receptor) and human medulloblastoma TE671 cells (with an $\alpha_1\beta_1\gamma\delta$ -nicotinic receptor).

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A.1.3.30

Assessment of Ganglion-Blocking Activity in the Isolated Bovine Retractor Penis Muscle

PURPOSE AND RATIONALE

The use of the bovine retractor penis muscle for the assessment of ganglion-blocking activity of neuromuscular blocking drugs has been recommended by Alaranta et al. (1990) and Klinge et al. (1993). Klinge and Sjöstrand (1974) performed not only extensive studies on the physiology and pharmacology of the retractor penis in the bull, but also discussed the various hypotheses on inhibitory and excitatory innervation of this muscle, which is present in many vertebrates such as horses, cats, dogs and rats, but not in men and rabbits. They also found that the effects on the isolated retractor penis muscle and on penile arteries are rather similar. The excitatory innervation was found to be predominantly α -adrenergic (Klinge et al. 1970; Klinge and Sjöstrand 1977) whereas other transmitters such as histamine and bradykinin were effective only in some species. Relaxation of the isolated retractor penis muscle could be elicited by nicotine and other nicotinic agonists (Klinge et al. 1988). In the studies on ganglion-blocking activity, strips of the retractor penis muscle are precontracted by 5-hydroxytryptamine. Relaxation induced by nicotine is antagonized by ganglion-blockers.

PROCEDURE

Retractor penis muscles are obtained from bulls of different breeds weighing 250–500 kg. Samples are dissected 10–30 cm distal to the points where the paired muscle bundles pass the anal orifice. Immediately after slaughter, the samples are freed from fat and other surrounding tissue and placed into Tyrode solution at 2–4°C. Strips, 15–25 mm in length and 2–3 mm wide, are prepared and mounted in 20-ml organ baths containing Tyrode solution at 35°C aerated with 95% O₂ and 5% CO₂. An equilibrium time of 2 to 4 h is allowed. During the equilibrium period washed are performed at about 60-min intervals. Changes in tension are recorded by means of Grass FT 03 force displacement transducers coupled to a polygraph.

A high-enough tone for studying the nicotine-induced relaxation, usually 8–15 g, is generated by adding 5-HP in a concentration between 0.1 and 6 μ M to the organ bath. Washing is performed 2 min after application of nicotine; 60–80 min later the tone is again raised and the application of nicotine is repeated. The effect of a neuromuscular blocking drug is studied only if the relaxations caused by nicotine in two consecutive controls are equal in size.

EVALUATION

The blocking activity of a certain concentration of a drug is expressed as % reduction in the relaxation of the muscle strip, according to the following equation:

$$\frac{A - B}{A} \times 100$$

Where *A* is the size of the control relaxation in millimeters, and *B* is the size of the relaxation of the blocking drug. In order to construct regression lines, the activity of four or five dose levels from the assumed linear part of the concentration-effect curve is studied. The activity of each dose level is studied in at least 5 strips obtained from different animals. *IC*₅₀ values are calculated from the regression lines. The parallelism of the regression lines is tested by covariance analysis.

CRITICAL ASSESSMENT OF THE METHOD

Molar potency ratios of known ganglion-blocking agents obtained with this method were compared with the results of other methods, such as inhibition of contraction of cat nictitating membrane evoked by preganglionic sympathetic stimulation (Bowman and Webb 1972; see 1.3.29), inhibition of nicotine-induced contraction of the isolated guinea pig ileum (Feldberg 1951), inhibition of contraction of guinea pig vas deferens evoked by preganglionic stimulation of the hypogastric nerve *in vitro* (Birmingham and Hussain 1980), depression of postganglionic action potentials evoked by preganglionic stimulation of the superior cervical ganglion of the rat *in vitro* (Quilliam and Shand 1964), induction of mydriasis in mouse by blocking the ciliary ganglion (Edge 1953). A fair but not a complete agreement between the results obtained with various methods was found.

MODIFICATIONS OF THE METHOD

Gillespie and Sheng (1990) studied the effects of pyrogallol and hydroquinone on the response to non-adrenergic, non-cholinergic nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles.

Parkkisenniemi and Klinge (1996) used samples of retractor penis muscles and penile arteries from bulls for functional characterization of endothelin receptors.

La et al. (1997) studied the inhibition of nitrenergic nerve-induced relaxations in rat anococcygeus and bovine retractor penis muscles by hydroxycobalamin.

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A.1.3.31

Angiotensin II Antagonism

PURPOSE AND RATIONALE

Angiotensin II antagonists can be tested in rats after elimination of cardiovascular reflexes by vagotomy and ganglionic blockade. Several angiotensin II

antagonists possess intrinsic agonistic activity. This can be tested by injection of various doses to the vagotomized, ganglion-blocked animal. The antagonistic activity of the angiotensin II antagonist can be evaluated by antagonism against graded doses of angiotensin II. The duration of activity can be tested during continuous infusion of angiotensin II.

PROCEDURE

Male Sprague-Dawley rats weighing about 300 g are used. They are anesthetized with 60 mg/kg pentobarbital sodium i.v. One carotid artery is cannulated and connected with a Statham transducer P 23 Db. Blood pressure is recorded on a polygraph. Both jugular veins are cannulated for application of test compounds and for infusion. Both vagal nerves are cut 3 mm dorsal of the larynx. For ganglionic blockade, 10 mg/kg pentolinium tartrate are injected intravenously. At least 5 animals are used for evaluation of one test drug.

Intrinsic Agonistic Activity

After the blood pressure has reached a constant value, doses of 1, 2, 4 and 16 µg/kg of the test compound are injected via the jugular vein. Blood pressure is recorded.

Antagonistic Activity

In 10 min intervals doses of 0.5; 1.0; and 2.0 µg/kg angiotensin II are injected to establish dose-response curves. After 10 min, continuous infusion is started of the potential angiotensin II blocker in a dosage of 10 µg/kg/0.1 ml/min. Ten min after beginning of the infusion, again doses of 0.5; 1.0; and 2.0 µg/kg angiotensin II are injected.

Duration of Activity

In this set-up, angiotensin II is administered as continuous infusion at a dosage of 1 µg/kg/0.02 ml/min. When blood pressure has reached an elevated steady state level, 0.1 mg/kg of the angiotensin II antagonist is administered.

Intensity and duration of the fall of blood pressure are recorded.

EVALUATION

Intrinsic Agonistic Activity

An increase of blood pressure indicates the intrinsic agonistic activity.

Antagonistic Activity

Increases of blood pressure after graduated doses of angiotensin II during the infusion is expressed as per-

centage of the increase before infusion. The results are compared with known angiotensin II antagonists.

CRITICAL ASSESSMENT OF THE METHOD

In this test not only potency and duration of activity but also the intrinsic agonistic activity of an angiotensin II antagonist can be tested.

MODIFICATIONS OF THE METHOD

Various other pharmacological models have been used to test angiotensin II antagonists:

Blood pressure in conscious unrestrained rats with chronically implanted catheters with normal blood pressure, spontaneous hypertension and chronic renal hypertension (Vogel et al. 1976; Chiu et al. 1989; Brooks et al. 1992; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Nagura et al. 1995; Nozawa et al. 1995; Renzetti et al. 1995; Wong 1995; Junggren et al. 1996),

Blood pressure in conscious spontaneously hypertensive and in anesthetized ganglion-blocked rats (Olins et al. 1993),

Blood pressure in pithed and in conscious renovascular hypertensive rats (Criscone et al. 1993; Wienen et al. 1993; Deprez et al. 1995; Kivlighn et al. 1995a; Kushida et al. 1995),

Blood pressure in rats after intracerebroventricularly injected angiotensin II (Vogel et al. 1976; Batt et al. 1988),

Blood pressure in conscious angiotensin I-infused and renin-dependent **hypertensive dogs** (Brooks et al. 1992; Cazaubon et al. 1993; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Keiser et al. 1995; Wong et al. 1995),

Blood pressure and heart rate in conscious sodium-depleted and sodium-repleted **cynomolgus monkeys** (Lacour et al. 1993; Cazaubon et al. 1993; Keiser et al. 1995),

Angiotensin II induced pressor responses in **marmosets** (Nagura et al. 1995),

Blood pressure and heart rate in conscious **rhesus monkeys** and anesthetized **chimpanzees** (Gabel et al. 1995; Kivlighn et al. 1995b; Kivlighn et al. 1995c).

Inhibition of angiotensin II-induced contraction in isolated **aorta** rings or strips from **rabbits** (Chui et al. 1989, 1990; Criscione et al. 1993; Cazaubon et al. 1993; Olins et al. 1993; Wienen et al. 1993; Aiyar et al. 1995; Caussade et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Kushida et al. 1995; Nagura et al. 1995; Renzetti et al. 1995; Wong et al. 1995), from **rats** (Nozawa et al. 1997), from **neonatal rats** (Keiser et al. 1993), from **guinea pigs** (Mizuno et al. 1995),

Inhibition of angiotensin II-induced contraction in isolated rat pulmonary artery (Chang et al. 1995),

Antagonism against angiotensin II in isolated strips of rabbit aorta, rabbit jugular vein, rabbit pulmonary artery, rat portal vein, rat stomach, rat urinary bladder, human urinary bladder, human colon, human ileum (Rhaleb et al. 1991),

Contractions of **guinea pig ileum in situ** (Khairallah and Page 1961),

Antagonism against angiotensin II in the **isolated rat uterus** (Wahhab et al. 1993),

Contractile force and prostaglandin E synthesis in electrically stimulated **rabbit isolated vas deferens** (Trachte et al. 1990),

Antagonism against angiotensin II-induced aldosterone release in **bovine adrenal glomerulosa cells**. (Criscione et al. 1993), and in rat dispersed adrenal capsular cells (Chang et al. 1995),

Antagonism against angiotensin II-induced inhibition of guanylate cyclase activity in the **rat pheochromocytoma cell line PC12W** (Brechler et al. 1993).

Brooks et al. (1995) compared the cardiovascular and renal effects of an angiotensin II receptor antagonist and captopril in **rats with chronic renal failure** induced by 5/6 nephrectomy. Under sodium pentobarbital anesthesia the right kidney was removed and approximately two thirds of the left kidney was infarcted by ligating two or three branches of the left renal artery.

Kim et al. (1997) studied the effects of an angiotensin AT₁ receptor antagonist on volume overload-induced cardiac gene expression in rats. An abdominal aortacaval shunt was prepared in 9-weeks old male Wistar rats under sodium pentobarbital anesthesia. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two thirds caudal to the renal artery and one third cephalic to the aortic bifurcation with a 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating the vena cava. After the aorta was clamped, the needle was withdrawn, and a drop of cyanoacrylate glue was used to seal the aortic puncture point. The patency of the shunt was verified visually by swelling of the vena cava and mixing of arterial and venous blood. The rats were treated either with vehicle or the angiotensin antagonist. Four days after the preparation of the AC shunt, 24 h-urine volume, electrolytes and aldosterone were measured. Six days after the AC shunt blood was collected by puncture of a tail vein and plasma renin activity and aldosterone were measured. Seven days

after AC shunt, hemodynamic studies were performed in pentobarbital anesthesia. Afterwards, the heart was rapidly excised, left and right atria and ventricles were separated and frozen in liquid nitrogen for the extraction and measurement of cardiac tissue RNA.

Shibasaki et al. (1997) tested the effect on the renin-angiotensin-aldosterone system in **conscious rats** after cannulation of the abdominal aorta under anesthesia 3–4 days before the experiment. After oral dosing of the angiotensin II receptor antagonist blood samples were withdrawn and plasma renin and aldosterone determined by radioimmunoassay.

Similar to the effects of ACE inhibitors, lifespan of hypertensive rats could be doubled by long-term treatment with an angiotensin II type 1 receptor blocker (Linz et al. 2000).

Ledingham and Laverty (1996) treated **genetically hypertensive New Zealand rats** with a specific AT₁ receptor antagonist via osmotic minipumps for several weeks and measured the effects on blood pressure, cardiac hypertrophy and the structure of resistance arteries.

Transgenic animals were recommended for further studies to influence the human renin-angiotensin system (Müller et al. 1995; Wagner et al. 1995; Bohlender et al. 1996).

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A.1.3.32

ACE Inhibition Measured in Vivo in the Rat

PURPOSE AND RATIONALE

The angiotensin-converting enzyme (ACE) is responsible for the cleavage of the almost inactive angiotensin I to the active angiotensin II. The same enzyme (kininase II) is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and diminution of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The cardiovascular system is sensitive to both peptides, reacting with an increase of blood pressure to angiotensin II and with a decrease to bradykinin. These

reactions can be used for quantitative determination of ACE inhibiting activity.

PROCEDURE

Male Sprague-Dawley rats weighing 300–400 g are used. The animals are anesthetized by i.p. injection of 70 mg/kg pentobarbital. After intubation of the trachea they are artificially respired with 30 strokes/min and a stroke volume of 6–8 ml. The right carotid artery is cannulated and blood pressure registered with a Statham-element (P 23 Db) and a polygraph. One jugular vein is cannulated for i.v. injections. After laparotomy a catheter is inserted into the duodenum for enteral administration and the wound closed again. Blood pressure is stabilized 30% below the normal level by i.m. injection of 5 mg/kg pentolinium. In order to prevent excessive mucus production in the bronchial system, 40 µg/kg atropine sulfate are injected intramuscularly.

Inhibition of Angiotensin I Cleavage

After stabilization of blood pressure, 310 ng/kg angiotensin I is injected intravenously in 0.1 ml saline. The injection is repeated in 5-min intervals until an identical pressure reaction occurs. The test compounds are administered at doses of 1 and 10 mg/kg intravenously or 25 mg/kg intraduodenally. 3 min after iv. injection or 10 min after i.d. administration, again 310 ng/kg angiotensin I is injected. Standards are ramipril, enalapril or captopril.

Potentiation of Bradykinin-Induced Vasodepression

A low dose of bradykinin has to be chosen in order to visualize the bradykinin potentiation. One µg/kg, eventually 3 µg/kg bradykinin are injected intravenously at 5 min intervals until a stable reaction is achieved. Three min after i.v. injection or 10 min after intraduodenal administration of the test substance, the bradykinin injection is repeated.

EVALUATION

Inhibition of Angiotensin I Cleavage

The diminution of the pressure reaction to angiotensin I after administration of a potential ACE inhibitor is the parameter for the activity of the new compound. The inhibition is calculated as percent of controls. Using various doses of the ACE inhibitor, dose-response curves can be established and *ID*₅₀ values be calculated.

Potential of Bradykinin-Induced Vasodepression

Potential of bradykinin induced vasodepression is expressed as percentage of controls. Using various doses of the test compound and the standard, dose-response curves can be established and potency ratios calculated.

CRITICAL ASSESSMENT OF THE METHOD

Both parameters, inhibition of angiotensin I response and potentiation of bradykinin-induced vasodepression have been proven as reliable parameters for evaluation of ACE inhibitors.

MODIFICATIONS OF THE METHOD

Natoff et al. (1981) used the ratio of responses to angiotensin I and angiotensin II in spontaneously hypertensive rats, either pithed or anesthetized with urethane, to determine the degree and the duration of effect of captopril.

Blood levels of angiotensin II can also be measured by radioimmunoassay.

Several studies in rats showed the beneficial effects of prolonged treatment with ACE inhibitors. Postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction was decreased by ACE inhibition (Linz et al. 1996).

Inhibition of angiotensin I-induced pressure response by administration of ACE-inhibitors can be measured not only in anesthetized rats, but also in anesthetized dogs, conscious rats and conscious dogs (Becker et al. 1984).

Life-long ACE inhibition doubles lifespan of hypertensive rats not only if the treatment is started at the age of one month (Linz et al. 1997), but ramipril also increases survival in old spontaneously hypertensive rats if treatment is started at the age of 15 months (Linz et al. 1999).

Panzenbeck et al. (1995) reported that captopril-induced hypotension is inhibited by the bradykinin blocker HOE 140 in Na⁺ depleted **marmosets**.

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A.1.3.33**Evaluation of Renin Inhibitors in Dogs****PURPOSE AND RATIONALE**

Highly specific inhibitors of the enzyme renin are considered to be potential antihypertensive agents. These agents cause a fall in blood pressure of sodium-deficient dogs and decrease plasma renin activity as well as angiotensin II level.

PROCEDURE**Animal Experiment**

Adult mongrel dogs (8–14 kg) of either sex are given water ad libitum and maintained on a low sodium diet for 1–2 weeks before the experiment. A single intramuscular injection of 5 mg/kg furosemide is given 48 h before the experiment. On the day of the experiment the dogs are anesthetized with sodium pentobarbital (30 mg/kg i.v.) and a cuffed endotracheal tube is positioned to allow artificial respiration. To measure arterial blood pressure a femoral artery is catheterized with polyethylene tubing. The right and left femoral veins are catheterized for drug administration and delivery of a maintenance infusion of sodium pentobarbital (5 mg/kg/h). Blood pressure is measured directly through the catheter, which is connected to a Gould-Statham pressure transducer. Blood samples are collected from the arterial catheter.

Increasing doses of the potential renin inhibitor are infused over 30 min followed by a 30 min recovery period. Immediately after the last recovery period, the dogs are given an i.v. infusion of the angiotensin receptor antagonist saralasin (20 µg/kg/min) for 30 min. For measurement of plasma renin activity and an-

giotensin II levels, the dogs are infused over a period of 30 min with the test compound and blood is withdrawn at 0, 15, 30, 60, 90, 120, 180, and 240 min after the start of the infusion. After the final blood drawing, 20 µg/kg/min saralasin is infused for 30 min.

Analytical Procedures

The antibody-trapping method is preferred to measure plasma renin activity (PRA). In this procedure PRA is determined at pH 7.4 by RIA quantification of angiotensin I (ANG I) generated and then trapped by excess anti-ANG I antibody (Poulsen and Jørgensen 1973; Nussberger et al. 1987). In tubes coated with rabbit anti-ANG I antibody (Gamma Coat™ ¹²⁵I Plasma renin activity RIA kit; Baxter Travenol Diagnostics) and incubated in an ice-water bath, 75 µl plasma are mixed with 7 µl 3 M TRIS base buffer (pH 7.2) containing 200 mM EDTA, and 3 µl 0.2 M TRIS base (pH 7.5) containing 3 g/L human serum albumin (fraction V, Sigma). Tubes are vortexed and incubated at 37°C for 60 min. The incubation is terminated by placing the tubes in an ice-water bath. Next, 75 µl of the TRIS albumin buffer are added, followed by 1 ml phosphate RIA buffer (Gamma Coat™) containing 15,000 cpm of ¹²⁵I ANG I. Standard ANG I (0.2–50 ng/ml) is also incubated at 37°C for 60 min with 10 µl TRIS/albumin buffer. In an ice-water bath, low renin plasma (75 µl) is added to the standards before the addition of a 1 ml tracer solution. Samples and standards are incubated for 24 h at 4°C. Tubes are then aspirated and counted in a gamma counter.

Levels of immunoreactive angiotensin II (ir-ANG II) are measured using a procedure described by Nussberger et al. (1985). Two–three ml of whole blood are collected in prechilled glass tubes containing 125 µl of the following “inhibitor” solution: 2% ethanol, 25 mM phenanthroline, 125 mM EDTA, 0.5 mM pepstatin A, 0.1 mM captopril, 2 g/l neomycin sulfate, and 0.1 mM of the renin inhibitor CGP 38560. The tubes are then centrifuged and the plasma quickly frozen in liquid nitrogen and stored at –70°C. For extraction of angiotensin peptides, Bond-Elut cartridges (Bond-Elut-pH) containing 100 mg phenylsilica are used, along with a Vac Elut SPS24 vacuum manifold (Analytichem; Harbor City, CA). Each cartridge is pre-conditioned with 1.0 ml methanol (HPLC grade) followed by 1.0 ml of water (HPLC grade) at a vacuum pressure of 5 mm Hg. One ml of the thawed sample is then applied to the cartridge and washed with 3 ml HPLC grade water. The angiotensin peptides retained at the columns are eluted with 0.5 ml methanol (HPLC grade, vacuum pressure less than 5 mm Hg)

into polypropylene tubes coated with a buffer containing 0.2 M TRIS, 0.02% NaN₃, and 2.5 mg/ml fatty acid-free bovine serum albumin (pH 7.4 with glacial acetic acid). The methanol is evaporated at 40°C and ir-ANG II measured using an antibody (IgG Corp., Nashville, TN) with greater than 1000-fold selectivity for ANG II.

EVALUATION

All data are expressed as mean ± SEM. The hypotensive responses after various doses of the renin antagonist are compared with the inhibition of plasma renin activity and the decrease of immunoreactive angiotensin II.

CRITICAL ASSESSMENT OF THE METHOD

The antibody-trapping method, reported here, gives a better correlation with the blood pressure lowering effect in dogs than the conventional method based on RIA for generated ANG I (Palmer et al. 1993).

MODIFICATIONS OF THE METHOD

Pals et al. (1990) described a rat model for evaluating inhibitors of human renin using anesthetized, nephrectomized, ganglion-blocked rats. The blood pressure rise induced by sustained infusion of renin was dose-dependently decreased by a renin inhibitor.

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A.1.3.34

Evaluation of Renin Inhibitors in Monkeys

PURPOSE AND RATIONALE

The renin-angiotensin-system as the main regulator of blood pressure can be influenced in several ways.

One approach involves the inhibition of renin. Renin is an aspartyl protease that hydrolyzes angiotensinogen to release the decapeptide angiotensin I, which is subsequently converted to angiotensin II by angiotensin-converting-enzyme. Sequencing of renin and angiotensinogen from various species revealed marked species differences for both the enzyme and the substrate. Inhibitors developed for human renin show a high specificity for primate renin and show only weak inhibition of renin from subprimate species. This means that the most common laboratory animals, such as rats and dogs, are not suitable for the *in vivo* evaluation of renin inhibitors. The marmoset was chosen by Wood et al. (1985, 1989) as a primate model.

PROCEDURE

Marmosets (*Callithrix jacchus*) of both sexes weighing between 300 and 400 g are fed a pellet diet supplemented with fruit. Two days prior to the experiment the animals are anesthetized and catheters are implanted in a femoral artery for measurement of blood pressure and in a lateral tail vein for injection or infusion of test substances. Thirty min before the experiment, the animals receive an intravenous injection of 5 mg/kg furosemide in order to stimulate renin release. During the experiment, the marmosets are sedated with diazepam (0.3 mg/kg i.p.) and kept in restraining boxes. Mean blood pressure is recorded continuously, and heart rate is measured at fixed intervals. The test compound or the standard are injected at various doses by intravenous infusion or administered orally.

EVALUATION

Blood pressure is recorded after 30 min of intravenous infusion and 30 min after stopping the infusion. Comparing the changes from pretreatment values after various doses, dose-response curves can be established.

MODIFICATIONS OF THE METHOD

Fischli et al. (1991) monitored arterial pressure in conscious and chronically instrumented monkeys using a telemetry system. One week before the experiment, the animals were anesthetized, and a 3F high fidelity pressure tip transducer (Millar Instruments, Inc.) was inserted into the abdominal aorta through the right femoral artery. Then the catheter was tunneled subcutaneously to the back of the monkey in the interscapular region. The proximal part of the catheter was connected to a transmitter located in a jacket worn by the monkey. The blood pressure was transmitted continu-

ously to a receiver, which transformed the signal to an analogue value of blood pressure.

Linz et al. (1994) reported on the effects of renin inhibitors in anesthetized rhesus monkeys weighing between 5 and 13 kg. The animals are sodium-depleted by administration of 10 mg/kg/day furosemide-Na for 6 consecutive days. At day 7, 10 mg/kg furosemide is given i.v. 30 min before the start of the experiment. Anesthesia is induced with 20 mg/kg ketamine-hydrochloride i.m. and continued with 40 mg/kg pentobarbitone-Na, slow i.v. drip. After completion of surgical procedures and after insertion of catheters under fluoroscopic control, the following hemodynamic parameters are measured: Pulse rate, and systolic and diastolic blood pressures are registered with a transducer (Statham P23 ID) in one femoral artery. A catheter tip manometer (Millar Instruments, Houston, Texas, USA) is introduced into the left ventricular cavity for the determination of left ventricular pressure. Contractility is electronically deduced from left ventricular pressure with appropriate amplifiers (Hellige GmbH, Freiburg, Germany). The electrocardiogram (ECG) from conventional lead II is taken using an ECG transducer (Hellige GmbH). Heart rate is measured from QRS-peaks using a biotachometer (Hellige GmbH). Cardiac output is determined using the thermodilution method. Thermodilution is integrated and converted to cardiac output readings by commercially available equipment (HMV 7905, Hoyer, Bremen). To determine cardiac output, 2 ml chilled 0–5°C isotonic glucose solution (5%) is injected rapidly into the right ventricle by a catheter via the right jugular vein. A thermistor is placed into the aortic arch via the right carotid artery.

Hemodynamics are monitored for 30 min following i.v. injection of various doses of the potential renin inhibitor. At the end of the experiments the ACE inhibitor ramiprilat 100 µg/kg is given i.v. to probe for an additional blood pressure lowering effect. Blood samples for the determination of ANG II concentration, renin inhibition and plasma drug levels are withdrawn at 10, 30 and 60 min after i.v. injection of the renin inhibitor. The volume is replaced by i.v. injections of isotonic glucose solution (5%). After all data and blood samples have been obtained, animals are sacrificed by an overdose of pentobarbitone-Na.

For experiments after intraduodenal administration sodium depletion and anesthesia are done as described above. A small side branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure measurements using a pressure transducer (P23 ID). Heart rate is determined from a conventional

ECG lead by a biotachometer. Blood samples are withdrawn via a catheter placed into the saphenous vein. A gastric fiberscope (Olympus XP10) is introduced into the duodenum under visual control and the renin inhibitor is administered intraduodenally through the service channel of the fiberscope in a volume of 5 ml. Blood samples are withdrawn before and at 15, 30, 45, 60, 90 and 120 min after intraduodenal administration.

Wood et al. (2005) tested an orally effective renin inhibitor (aliskiren) in marmosets. Blood pressure and heart rate were measured by telemetry in conscious animals moving freely in their home cages. Pressure transmitters (AM Unit, model TA11PA-C40 Data Sciences, USA) were implanted into the peritoneal cavity under aseptic conditions and light anesthesia. The sensor catheter was placed in the aorta below the renal artery pointing upstream.

CRITICAL ASSESSMENT OF THE METHOD

Due to the high species specificity of renin and its substrate, angiotensinogen, renin inhibitors for treatment of hypertension have to be tested in primate models. The marmoset as well as the rhesus monkey have been proven to be suitable models.

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A.1.3.35

Penile Erection in Rabbits

PURPOSE AND RATIONALE

The discovery of inhibitors of phosphodiesterase as effective drugs for patients with erectile dysfunction (Klotz et al. 2001; Porst et al. 2001) has stimulated the use of appropriate *in vivo* animal models. In particular, rabbits have been recommended as models for impotence research (Bischoff and Schneider 2000, 2001; Bischoff 2001; Bischoff et al. 2001; Saenz de Tejada et al. 2001) confirming earlier work in this area (Thielen et al. 1969; Sjöstrand and Kline 1979; Naganuma et al. 1993; Lin and Lin 1996).

PROCEDURE

Adult male Chinchilla rabbits weighing 3.5–4.5 kg are housed in individual cages for at least 1 week after arrival, at room temperature with water and food *ad libitum*.

For the study, an indwelling catheter filled with saline is inserted into a marginal ear vein and taped in position. The drugs are injected into the ear vein, followed by a small volume of saline. The time is noted and at appropriate times the animal gently removed from the cage and held by one research worker. The rabbit penis is not visible when it is not erect (Naganuma et al. 1993). However, when erection occurs, it is possible to examine the pudendal area and measure the length of the uncovered penile mucosa with sliding calipers.

EVALUATION

Penile erection is evaluated by measuring the length to the nearest millimeter of the uncovered penile mucosa with a sliding caliper at 5, 10, 15, 30, 50, 60, 90, and 120 min after administration of the test compounds and continued hourly for up to 5 h. Mean values are calculated and results expressed as means \pm SEM. The area under the curve is calculated by an integration program.

MODIFICATIONS OF THE METHOD

Choi et al. (2002) compared the efficacy of verdenafil and sildenafil in facilitating penile erection in **anesthetized rabbits**. Penile erections were elicited by submaximal pelvic nerve stimulation every 5 min for 30 min. Response was assessed by continuously

recording intracavernosal pressure and systemic arterial pressure.

Min et al. (2000) tested the augmentation of pelvic-nerve-mediated sexual arousal in anesthetized **female rabbits** by sildenafil. The following parameters were measured before, during and after pelvic nerve stimulation at 4, 16, and 32 Hz: (1) hemoglobin concentration and oxygen saturation in female genital (vaginal, labial, clitoral) tissues by laser oximetry; (2) clitoral blood flow by laser Doppler flowmetry; (3) vaginal luminal pressure by a balloon catheter pressure transducer; (4) vaginal lubrication by tampon.

Carter et al. (1998) tested the effect of the selective phosphodiesterase type 5 inhibitor sildenafil on erectile dysfunction in **pentobarbital-anesthetized dogs**. Increases in intracavernosal pressure in the corpus cavernosum and penile blood flow were induced by pelvic nerve stimulation over a frequency range of 1–16 Hz. The effects of increasing doses of sildenafil on electric-stimulated intracavernosal pressure, penile blood flow, blood pressure, and heart rate were evaluated.

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A.1.4

Cardiovascular Safety Studies

See:

Brian D. Guth

Methods in Cardiovascular Safety Pharmacology

Chapter I. D.

In:

Drug Discovery an Evaluation

Safety and Pharmacokinetic Assays

By: H.G: Vogel (Ed), F.J. Hock, J. Maas, D. Mayer (CoEds)

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A.2 Methods to Induce Experimental Hypertension

A.2.0.1

Acute Renal Hypertension in Rats

PURPOSE AND RATIONALE

Since the classical experiments of Goldblatt et al. (1934) there is clear evidence that the ischemia of the kidneys causes elevation of blood pressure by activation of the renin-angiotensin system. The principle can be used both for acute and chronic hypertension. In rats acute renal hypertension is induced by clamping the left renal artery for 4 h. After reopening of the vessel, accumulated renin is released into circulation. The protease renin catalyzes the first and rate-limiting step in the formation of angiotensin II leading to acute hypertension. The test is used to evaluate antihypertensive activities of drugs.

PROCEDURE

Male Sprague-Dawley rats weighing 300 g are used. The animals are anesthetized by intraperitoneal injection of 100 mg/kg hexobarbital sodium. A PVC-coated Dieffenbach clip is placed onto the left hilum of the kidney and fixed to the back muscles. The renal artery is occluded for 3.5–4 h.

3.5 h following the surgery, the animals are anesthetized by intraperitoneal injection of 30–40 mg/kg pentobarbital sodium. The trachea is cannulated to facilitate spontaneous respiration. To measure systolic and diastolic blood pressure, the cannula in the carotid artery is connected to a pressure transducer (Statham P 23 Db).

For administration of the test compound, a jugular vein is cannulated.

Following a stable blood pressure state, ganglionic blockade is performed with pentolinium (10 mg/kg i.v.). After obtaining stable reduced blood pressure values, the renal arterial clip is removed. This leads to a rise in blood pressure as a consequence of elevated plasma renin level. Within 15 min a stable hypertension is achieved (control = 100%).

The test substance is then administered by intravenous injection at doses of 10 and 100 µg/kg.

Blood pressure is monitored continuously until a renewed increase to the starting level is obtained.

Ten–twelve animals are used per compound.

EVALUATION

Increase in blood pressure after reopening of the renal artery and reduction in blood pressure after administration of the test drug are determined [mm Hg]. Percent inhibition of hypertensive blood pressure values under drug treatment are calculated as compared to pretreatment hypertension values. Duration of the effect is determined [min]. Statistical significance is assessed by the paired *t*-test.

MODIFICATIONS OF THE METHOD

A sharp and transient in systemic arterial blood pressure associated with reflex bradycardia can be elicited by injection of 5-hydroxytryptamine, cyanide, nicotine or lobeline into the coronary artery blood stream of dogs (Berthold et al. 1989). The phenomenon is named the cardiogenic hypertensive chemoreflex and 5-HT proved to be the most powerful agent for its initiation (James et al. 1975).

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A.2.0.2

Chronic Renal Hypertension in Rats

PURPOSE AND RATIONALE

On the basis of the findings of Goldblatt et al. (1934) that ischemia of the kidneys induces hypertension, various modifications of the technique have been described for several animal species. One of the most effective modifications in rats is the so called 1-kidney-1-clip method.

PROCEDURE

Male Sprague-Dawley rats weighing 200–250 g are anaesthetized with 50 mg/kg i.p. pentobarbital. The fur on the back is shaved and the skin disinfected. In the left lumbar area a flank incision is made parallel to the long axis of the rat. The renal pedicle is exposed with the kidney retracted to the abdomen. The renal

artery is dissected clean and a U-shaped silver clip is slipped around it near the aorta. Using a special forceps (Schaffenburg 1959) the size of the clip is adjusted so that the internal gap ranges from 0.25–0.38 mm. The right kidney is removed through a flank incision after tying off the renal pedicle. The skin incisions are closed by wound clips.

4–5 weeks after clipping blood pressure is measured and rats with values higher than 150 mm Hg selected for the experiments. Blood pressure readings are taken on each of 3 days prior to drug treatment. Drugs are administered orally in volumes of 10 ml/kg. The rats are divided into 4 animals per dose and each animal is used as his own control. Compounds are administered for 3 days and predrug and 2 h postdrug blood pressure readings are taken.

EVALUATION

Changes in systolic blood pressure are expressed in mm Hg. Activity is determined by comparing treatment blood pressure values with the control blood pressure value (Day 1, predrug blood pressure). Comparisons are made using the paired *t*-test for evaluation of statistical significance.

MODIFICATIONS OF THE METHOD

Duan et al. (1996) induced renal hypertension in male Hartley guinea pigs by a two-step procedure consisting of ligation of the left caudal renal artery and right nephrectomy. Arterial blood pressure and heart rate were monitored in conscious animals. ACE-inhibitors reduced blood pressure in sham-operated and in renal hypertensive guinea pigs, whereas renin inhibitors were effective only in renal hypertensive animals.

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A.2.0.3

Chronic Renal Hypertension in Dogs

PURPOSE AND RATIONALE

Production of hypertension by clamping renal arteries has been first described by Goldblatt et al. (1934) in dogs. Later on, the method has been modified, e. g., as the “wrapping” technique (Abram and Sobin 1947).

PROCEDURE

Dogs weighing 8–12 kg are anesthetized with i.v. injection of 15 mg/kg thiopental. Anesthesia is maintained with a halothane-oxygen mixture. Under aseptic conditions, a midline abdominal incision is made. One kidney is exposed and wrapped in cellophane and then replaced. The contralateral kidney is exposed. The artery, vein and ureter are ligated and the kidney is removed. The abdomen is closed by sutures and clips. On the day of surgery and for 3 days following, the dogs are given antibiotics. Body temperature is measured twice daily for 4 days following surgery.

Six weeks following surgery, blood pressure is measured using a tail-cuff method. For recording, the tail-cuff is attached to a polygraph. Only animals with a systolic blood pressure higher than 150 mm Hg are considered to be hypertensive and can participate in studies evaluating potential antihypertensive compounds.

For the experiment, blood pressure is recorded either by the indirect tail-cuff method or by direct measurement via an implanted arterial cannula. On day 1 readings are made every 2 h, just before, and 2 and 4 h after oral treatment with the potential antihypertensive compound. Drug administration is repeated for 5 days. On days 3 and 5 blood pressure readings are taken before and 2 and 4 h after treatment. At least 3 dogs are used per dose and compound.

EVALUATION

The starting value is the average of the 2 readings before application of the drug. Each of the following readings is subtracted from this value and recorded as fall of blood pressure at the various recording times.

MODIFICATIONS OF THE METHOD

Renal hypertension in rats has been achieved by many modifications of the method (Stanton 1971) such as the

technique according to Grollman (1944). The kidney is exposed through a lumbar incision, the renal capsule is removed by gentle traction, and a figure-8 ligature is applied being tight enough to deform the kidney but not tight enough to cut the tissue.

Renal hypertension may be induced in the **rat** by encapsulating both kidneys with latex rubber capsules (Abrams and Sobin 1947). Moulds are formed from plastic using a rat kidney as a model. The capsules are prepared by dipping the moulds in liquid latex allowing them to dry in the air. Three applications of latex are applied before the capsules are toughened by placing them under warm running tap water. The kidney is exposed by lumbar incision, the renal capsule gently removed and the capsule applied.

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A.2.0.4

Neurogenic Hypertension in Dogs

PURPOSE AND RATIONALE

Vasodilator and depressor reflexes, originating in the baroreceptor areas of the carotid sinus and aortic arch, play an important part in the regulation of blood pressure. Stimulation of the afferent buffer fibres exerts an inhibitory influence on the vasomotor center, and their sectioning leads to a persistent rise in blood pressure. In this way, acute neurogenic hypertension can be induced in dogs.

PROCEDURE

Adult dogs of either sex weighing 10–15 kg are anesthetized using 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital and 60 mg/kg sodium pentobarbital i.v. A femoral vein and artery are cannulated using

polyethylene tubing to administer compounds i.v. and record arterial pressure and heart rate, respectively. Left ventricular pressure and dp/dt are recorded via the left common carotid artery (post-deafferentation) using a Millar microtip pressure transducer. P_{max} is recorded by speeding up the chart paper. Cardiac output is determined by introducing a Swan-Ganz catheter into the right heart and pulmonary artery via a jugular vein. Five ml of cold 5% dextrose is injected into the right atrium and an Edwards Cardiac output computer is used to calculate the cardiac output from the temperature change in the pulmonary artery. All recordings are made with a polygraph.

Both of the carotid arteries are cleared up to the bifurcation of the internal and external carotid arteries. The carotid sinus nerves are isolated, ligated and sectioned and a bilateral vagotomy is performed to produce neurogenic hypertension (mean arterial pressure more than 150 mm Hg). The dog is allowed to equilibrate for approximately 30 min and a bolus of the test compound is administered by intravenous injection. Heart rate, arterial pressure, left ventricular pressure, P_{max} and dp/dt are monitored for 90 min. A minimum of 3 dogs are used for each compound.

EVALUATION

Changes of the cardiovascular parameters are expressed as percentage of the values before administration of the drug.

MODIFICATIONS OF THE METHOD

Neurogenic hypertension through baroreceptor denervation has also been described in **rabbits** (Angell-James 1984) and in rats (Krieger 1984).

CRITICAL ASSESSMENT OF THE METHOD

The neurogenic hypertension is useful for acute experiments. However, it is less useful for chronic experiments since the elevated blood pressure caused by buffer nerve section is more labile than that caused by renal ischemia.

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A.2.0.5

DOCA-salt Induced Hypertension in Rats

PURPOSE AND RATIONALE

Mineralocorticoid-induced hypertension is thought to be due to the sodium retaining properties of the steroid causing increases in plasma and extracellular volume. The hypertensive effect is increased by salt loading and unilateral nephrectomy in rats.

PROCEDURE

Male Sprague Dawley rats weighing 250–300 g are anesthetized with ether. Through a flank incision the left kidney is removed. The rats are injected twice weekly with 20 mg/kg s.c. desoxycorticosteroneacetate in olive oil for 4 weeks. Drinking water is replaced with a 1% NaCl solution. Blood pressure starts to rise after one week and reaches systolic values between 160 and 180 mm Hg after 4 weeks.

MODIFICATIONS OF THE METHOD

The regimen to induce DOCA-salt hypertension has been modified by many authors (Stanton 1971).

DOCA pellets (Peterfalvi and Jequier 1960; Passmore and Jimenez 1990) or implants in silastic devices (Ormsbee and Ryan 1973; King and Webb 1988) were used instead of repeated injections.

DOCA-salt hypertension can also be achieved without nephrectomy (Bockman et al. 1992).

Using kininogen-deficient Brown Norway Katholiek (BN-Ka) rats, Majima et al. (1991, 1993) showed suppression of rat desoxycorticosterone-salt hypertension by the kallikrein-kinin system.

Li et al. (1996) examined small-artery structure on a wire myograph and quantified endothelin-1 messenger RNA by Northern blot analysis in DOCA-salt hypertensive rats after administration of an ACE-inhibitor, a calcium channel antagonist and a nitric oxide synthase inhibitor.

Ullian (1997) described the **Wistar-Furth rat** as a model of mineralocorticoid resistance. These rats de-

veloped two-kidney, one-clip hypertension to the same degree as did Wistar rats and reacted to glucocorticoid treatment with a rapid onset of hypertension, but were resistant to the development of DOCA-NaCl hypertension.

Studies in DOCA-salt hypertensive mice were reported by Gross et al. (1998, 1999), Honeck et al. (2000), Peng et al. (2001).

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A.2.0.6

Fructose Induced Hypertension in Rats

PURPOSE AND RATIONALE

Increases in dietary carbohydrate intake can raise blood pressure in experimental animals. The increased intake of either sucrose or glucose was shown to enhance the development of either spontaneous hypertension or salt hypertension in rats (Hall and Hall 1966; Preuss and Preuss 1980; Young and Landsberg 1981). Hwang et al. (1987) first reported that hypertension could be induced in normal rats by feeding a high-fructose diet. Fructose feeding was also found to cause insulin resistance, hyperinsulinemia, and hypertriglyceridemia in normal rats (Zavaroni et al. 1980; Tobey et al. 1982). Dai and McNeill (1995) studied the concentration- and duration-dependence of fructose-induced hypertension in rats.

PROCEDURE

Groups of 8 male Wistar rats weighing 210–250 g are used. The are housed two per cage on a 12-h light 12-h dark cycle and are allowed free access to standard laboratory diet (Purina rat chow) and drinking fluid. Drinking fluid consists either of tap water or 10%-fructose solution. Body weight, food intake and fluid intake of each rat are measured every week during treatment. Using the tail-cuff method, systolic blood pressure and pulse rate is measured before and every week during treatment. Blood samples are collected before and every second week during treatment for determination of plasma glucose, insulin, and triglycerides.

EVALUATION

Since maximum effects on the chosen parameters are achieved after 6 weeks, the duration of treatment can

be limited to this time. Statistical analysis is performed using a one-way or two-way analysis of variance, followed by the Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Reaven et al. (1988, 1989) found an attenuation of fructose-induced hypertension by exercise training and an inhibition by somatostatin treatment.

Brands et al. (1991, 1992) found an increase of arterial pressure during chronic hyperinsulinemia in conscious rats.

Hall et al. (1995) reported the effects of 6 weeks of a high-fat diet on cardiovascular, renal, and endocrine functions in chronically instrumented conscious **dogs**. Body weight increased by approximately 16.9 kg, whereas MAP, cardiac output, and heart rate increased by 28%, 77%, and 68%, respectively.

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A.2.0.7**Genetic Hypertension in Rats****SURVEY**

Inherited hypertension in rats has been described by Smik and Hall 1958; Phelan and Smirk 1960; Laverty and Smirk 1961; Phelan 1968 as **genetically hypertensive (GH) rats** (Simpson and Phelan 1984).

Okamoto et al. (1963, 1966) reported the development of a strain of spontaneously hypertensive rats from mating one Wistar male rat with spontaneously occurring high blood pressure with a female with slightly elevated blood pressure. By inbreeding over several generations a high incidence of hypertension with blood pressure values of 200 mm Hg or more was achieved. These strains were called "**Spontaneously hypertensive rats (Akamoto-Aoki)**" = **SHR** or "**Wistar-Kyoto rats**" = **WKY**. Hypertension in these rats is clearly hereditary and genetically determined, thus comparable to primary hypertension in humans. Cardiac hypertrophy (Sen et al. 1974) and cellular ionic transport abnormalities have been observed (Yamori 1984).

Inbred strains being **salt-hypertension-sensitive** and **salt-hypertension-resistant (RD)** have been developed by Dahl et al. (1962, 1963), Rapp (1984), Cicila et al. (1993). Inoko et al. (1994) reported the transition from compensatory hypertrophy to dilated, failing left ventricles in Dahl salt-sensitive rats.

Two strains of rats with inbred dissimilar sensitivity to DOCA-salt hypertension ("**Sabra strain**") have been separated by Ben-Ishay et al. (1972, 1984).

Another hypertensive strain derived from Wistar rats was produced by brother-sister mating in the group of Bianchi et al. (1974, 1986) at the University of Milan called "**Milan hypertensive strain**" = **MHS**. These rats show a cell membrane defect resulting in abnormal kidney function. Salvati et al. (1990) studied the diuretic effect of bumetanide in isolated perfused kidneys of Milan hypertensive rats.

Furthermore, the "**Lyon**" strains of hypertensive, normotensive and low-blood-pressure rats were developed (Dupont et al. 1973; Vincent et al. 1984; Dubay et al. 1993). These rats show a genetically determined defect in central nervous function.

Spontaneously hypertensive rats which develop failure before 18 months have been selectively bred. Several substrains of spontaneous hypertensive rats were separated by the group of Okamoto et al. (1974) including the **stroke-prone strain SHR=SHRSP**. These rats have an increased sympathetic tone and show a high incidence of hemorrhagic lesions of

the brain with motor disturbances followed by death (Yamori 1984; Feron et al. 1996).

A strain of obese spontaneously hypertensive rats has been described by Koletsky (1975), Ernberger et al. (1993).

With new techniques of genetic engineering, **transgenic rats with hypertension** could be created. Increase of blood pressure of spontaneously hypertensive rat is determined by multiple genetic loci (Deng and Rapp 1992; Dubay et al. 1993). With new technology not only these loci could be defined but also new models in hypertension research and models to detect antihypertensive drugs could be established (Bohlender et al. 1997; Pinto et al. 1998).

Mullins et al. (1990) reported fulminant hypertension in transgenic rats harboring the mouse Re-2 gene.

A **rat strain TGR(mREN2)27** as a monogenetic model in hypertension research was described by Peters et al. (1993), Lee et al. (1996), Langheinrich et al. (1996), and Ohta et al. (1996).

Bohlender et al. (1996) reconstructed the human renin-angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using Alzet pumps.

Zolk et al. (1998) described the effects of quinapril, losartan and hydralazine on cardiac hypertrophy and β -adrenergic neuroeffector mechanisms in transgenic TGR(mREN2)27 rats.

CRITICAL ASSESSMENT OF THE METHOD

The use of spontaneously hypertensive rats to detect potential antihypertensive compounds is well established. On the basis of available data no preference can be given to a particular strain. The most abundant experience has been gained with the Wistar-Kyoto strain. Transgenic rats with well defined genomes are gaining more importance.

MODIFICATIONS OF THE METHOD

Pijl et al. (1994) described streptozotocin-induced diabetes mellitus in spontaneously hypertensive rats as a pathophysiological model for the combined effects of hypertension and diabetes.

Rosenthal et al. (1997) used rats of the Cohen-Rosenthal diabetic hypertensive strain to examine the effects of an ACE-inhibitor, an ATII antagonist and a calcium antagonist on systolic pressure and spontaneous blood glucose levels.

Holycross et al. (1997) used hypertensive SHHF/Mcc-facp rats to study plasma renin activity during development of heart failure.

Linz et al. (1997) compared the outcome of lifelong treatment with the ACE inhibitor ramipril in young prehypertensive stroke-prone spontaneously hypertensive rats and age-matched normotensive Wistar-Kyoto rats. Lifelong ACE inhibition doubled the lifespan in hypertensive rats matching that of normotensive rats.

Studies in **genetically hypertensive mice** were reported by Rosenberg et al. (1985), Hamet et al. (1990) Meneton et al. (2000). Ohkubo et al. (1990) generated transgenic mice with elevated blood pressure by introduction of the rat renin and angiotensinogen genes.

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A.2.0.8

Hypertension Induced by Chronic NO-Synthase Inhibition

PURPOSE AND RATIONALE

Tonic basal release of nitric oxide (NO) by vascular endothelial cells controls blood pressure in the basal state. Chronic blockade of NO synthesis in the rat produces systemic hypertension and glomerular damage (Baylis et al. 1992). This was recommended by Ribeiro et al. (1992) as a model of hypertension. Yang et al. (1996) found an increase of vascular angiotensin II receptor expression after chronic inhibition of NO synthase in spontaneously hypertensive rats. The detrimental sequels of chronic NO synthase inhibition in rats can be inhibited by treatment with ACE inhibitors (Hropot et al. 1994; Küng et al. 1995). Hsieh et al. (2004) reported that NO inhibition by L-NAME accelerates hypertension and induces perivascular inflammation in rats.

Hropot et al. (2003) reported that angiotensin II subtype AT₁ receptor blockade prevents hypertension and renal insufficiency induced by chronic NO synthase inhibition in rats.

PROCEDURE

Male Wistar rats at an age of 7–8 weeks weighing 210 ± 10 g were placed at random in metabolic cages,

divided in four to six groups of six to eight rats each. Group 1 (control) had free access to tap water and food. Groups 2–4 were treated with 0.02% L-NAME water solution for 6 weeks in a daily dose of 25 mg/kg. Groups 3 and 4 received the angiotensin receptor antagonists fonsartan (10 mg/kg) or lorasatan (30 mg/kg) for 6 weeks daily per stomach tube. Groups 5 and 6 received fonsartan and lorasatan alone. At the end of the study, 24-h urine samples were collected and retrobulbar blood samples were taken in short inhalation anesthesia. Plasma values of creatine, PRA and electrolytes were determined. For clearance evaluation rats were anesthetized with 50 mg/kg thiopentone i.p. In order to determine glomerular filtration rate and renal plasma flow, clearances of inulin and *para*-aminohippurate were performed. After the clearance experiments, the rats were sacrificed and hearts and kidneys removed. Hearts were perfused in the isolated working heart preparation via the aorta with modified Krebs-Henseleit buffer containing solvents or drugs. After a pre-ischemic period of 20 min, acute regional myocardial ischemia was produced by clamping the left coronary artery close to its origin for 15 min (ischemic period). Thereafter, the clip was removed and changes during reperfusion were monitored (reperfusion period). The following cardio-dynamic and cardio-metabolic parameters were measured: incidence and duration of ventricular fibrillation, left ventricular pressure, contractility (dP/dt max), heart rate and coronary flow; in the coronary effluent lactate dehydrogenase, creatine kinase and lactate; in the myocardial tissue lactate, glycogen, ATP and creatine phosphate. Left ventricular pressure was measured with a Statham pressure transducer (P 23 DB) which on differentiation yielded LV dP/dt max and heart rate. Coronary flow was determined by electromagnetic flow probes in the aortic cannula. For the determination of lactate release, lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the perfusate samples were taken from the coronary effluent and analyzed spectrophotometrically.

EVALUATION

Results are presented as arithmetical means \pm SEM. A one-way ANOVA was calculated with SYSTAT for Windows (SYSTAT, Evanston, Ill., USA) followed by multiple pairwise comparisons according to Tukey.

MODIFICATIONS OF THE METHOD

Arnal et al. (1993) measured cardiac weight of rats in hypertension induced by NO synthase blockade.

Linz et al. (1999) reviewed the interactions between ACE, kinins and NO.

Sampaio et al. (2002) reported that hypertension plus diabetes mimics the cardiomyopathy induced by NO inhibition in rats.

Rossi et al. (2003) found that chronic inhibition of NO synthase induces hypertension and cardiomyocyte mitochondrial and myocardial remodeling in the absence of hypertrophy.

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A.2.0.9

Pulmonary Hypertension Induced by Monocrotaline

PURPOSE AND RATIONALE

The pyrrolizidine alkaloid monocrotaline, derived from *Crotalaria spectabilis*, is hepatotoxic and pneumotoxic in the rat. A single injection of monocrotaline

leads to progressive pulmonary hypertension resulting in right ventricular hypertrophy and cardiac failure (Gillespie et al. 1986, 1988; Todorovich-Hunter et al. 1988). Pathologic changes and hemodynamic changes associated with monocrotaline administration include blebbing of the lung, degeneration and fragmentation of endothelial cells, perivascular edema, extravasation of red blood cells, and muscularization of the pulmonary arteries and arterioles (Valdiva et al. 1967; Lalach et al. 1977; Huxtable et al. 1978; Hislop and Reid 1979; Meyrick and Reid 1979; Meyrick et al. 1980; Ghodsi and Will 1981; Hilliker et al. 1982; Sugita et al. 1983; Hilliker and Roth 1985; Stenmark et al. 1985; Altieri et al. 1986; Molteni et al. 1986; Lai et al. 1996). Rats given monocrotaline develop severe right ventricular hypertrophy often accompanied by ascites and pleural effusions (Ceconi et al. 1989).

Amelioration by angiotensin-converting enzyme inhibitors and by penicillamine has been demonstrated (Molteni et al. 1985, 1986).

PROCEDURE

Treatment of male Sprague Dawley rats weighing 200–225 g with the test drug (angiotensin converting enzyme inhibitor or vehicle) is started one week prior to a single subcutaneous injection of 100 mg/kg monocrotaline up to sacrifice 4, 7, or 14 days later by pentobarbital anesthesia and exsanguination. Heart and lungs are excised from thoracic cavity. After removing atria from the heart, the right ventricle is separated from the left ventricle plus septum which are blotted and weighed separately. Left lung is blotted, weighed, minced and reweighed after drying at room temperature for 14 days. Three pulmonary artery segments, main pulmonary artery, right extrapulmonary artery and an intrapulmonary artery from the from the right lower lobe, are isolated for study of vascular responsiveness. Cylindrical segments of each vessel are suspended between stainless steel hooks in 10-ml isolated tissue baths containing modified Krebs-Henseleit buffer aerated with 95% O₂/5% CO₂ at 37°C. At the end of each experiment, vessel segments are blotted and weighed and their dimensions measured. Cross-sectional area of each artery is determined from tissue weight and diameter.

Arteries are equilibrated for 1 h at 1 g of passive applied load and then are made to contract to KCl (6×10^{-2} M). After washout, the procedure is repeated with applied loads increased by 1 g increments. Responses are normalized to the maximum active force development generated by an artery in each experiment and the data are plotted as a function of ap-

plied force. Changes in isometric force are monitored through force displacement transducers (Grass FT03) and recorded on a polygraph.

Responsiveness to contractile and relaxant agonists is assessed in pulmonary arteries from saline- and monocrotaline-treated rats both in verum- and placebo-treated groups. Cumulative concentration-response curves to hypertonic KCl, angiotensin II and norepinephrine are generated sequentially in vessels at resting tone. Arteries are then contracted submaximally with norepinephrine and cumulative concentration-response curves to the vasorelaxants isoproterenol and acetylcholine are determined.

EVALUATION

Contractions are expressed as active tension development, force generated per cross-sectional area and relaxations are normalized to precontraction tone. Both contractile and relaxation responses are plotted as a function of the negative logarithm of agonist concentration. Differences in mean responses are compared by a *t*-test for grouped data.

MODIFICATIONS OF THE METHOD

Molteni et al. (1986) treated rats continuously with monocrotaline in the drinking water at a concentration of 2.4 mg/kg/day for a period of 6 weeks. Test rats received an ACE-inhibitor during this time in the drinking water and controls the vehicle only. At the end of the experiment, hearts and lungs were weighed and examined by light and electron microscopy.

Madden et al. (1995) determined L-arginine-related responses to pressure and vasoactive agents in monocrotaline-treated rat pulmonary arteries.

Ono et al. (1995) studied the effects of prostaglandin E₁ (PGE₁) on pulmonary hypertension and lung vascular remodelling in the rat monocrotaline model of human pulmonary hypertension.

Yamauchi et al. (1996) studied the effects of an orally active endothelin antagonist on monocrotaline-induced pulmonary hypertension in rats.

Gout et al. (1999) evaluated the effects of adrenomedullin in isolated vascular rings from rats treated with monocrotaline (60 mg/kg s.c.) causing pulmonary hypertension and ventricular hypertrophy within 3 to 4 weeks.

Kanno et al. (2001) studied the effect of an angiotensin-converting enzyme inhibitor on pulmonary arterial hypertension and endothelial nitric oxide synthase expression in monocrotaline-treated rats. For evaluation of right ventricular hypertrophy as a result of pulmonary arterial hypertension, multislice spin-

echo MRI images were acquired at 8–12 time points in a cardiac cycle with respiratory and ECG gating (Kanno et al. 2000) at 2, 3, 4, and 5 weeks after monocrotaline treatment.

Kang et al. (2003) reported that a phosphodiesterase-5 inhibitor attenuated monocrotaline-induced pulmonary hypertension in rats.

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A.2.0.10

Portal Hypertension in Rats

PURPOSE AND RATIONALE

Portal hypertension is associated with hyperdynamic splanchnic circulation and reduced vascular resistance (Vorobioff et al. 1983). Tanoue et al. (1991) developed a method for inducing portal hypertension and esophageal varices in rats – partial ligation of the portal vein after devascularization of the circumference of the left renal vein and complete ligation of the portal vein on the fifth day thereafter. Tsugawa et al. (2000)

used this model to study the role of nitric oxide and endothelin-1 in rat portal hypertension.

PROCEDURE

Male Sprague-Dawley rats were anesthetized with 50 mg/kg Nembutal intraperitoneally. The portal vein was isolated and stenosis created by a single ligature of 3–0 silk placed around the portal vein and a 20-gauge blunt-tipped needle after devascularization of the left renal vein. This devascularization is indispensable in preventing the development of excess collateral vessels, which inhibit the formation of esophageal varices and the portal hypertensive state. The needle was then removed from the ligature. In addition, 3–0 silk was also placed at the area of partial ligation (loose ligation), and both ends were then drawn out through the abdominal wall. Five days after the operation, the ends of the silk that had been placed in the flank were simultaneously pulled to induce complete portal vein ligation. Two weeks later, this portal hypertension model was completed.

Portal venous pressure, blood flow volume in the intra-abdominal viscera, plasma NO and plasma endothelin-1 were measured.

EVALUATION

Results were expressed as mean \pm standard deviation. The Student's *t*-test was used to determine significance between portal hypertension rats and sham-operated controls.

MODIFICATIONS OF THE METHOD

Portal hypertension by portal vein ligation without devascularization of the left renal vein was used by Lee et al. (1985), Braillon et al. (1986), Oren et al. (1995), Fernandez et al. (1996), Moreno et al. (1996), Connolly et al. (1999), Hilzenrat et al. (1999), Chagneau et al. (2000), Yu et al. (2000), and Sakurabayashi et al. (2002).

Dieguez et al. (2002) used a surgical technique based on the development of a triple stenosing ligation to worsen the complications inherent to the prehepatic chronic portal hypertension.

Jaffe et al. (1994) and Li et al. (1998) used injection of different sized microspheres into the portal vein of male Wistar rats to induce portal hypertension.

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A.3 Coronary (Cardiac) Drugs

A.3.1 Isolated Organs

A.3.1.1 Heart-Lung Preparation

PURPOSE AND RATIONALE

The isolated heart-lung of the dog was introduced by Knowlton and Starling (1912). Since then, the dog model has been used for many physiological and pharmacological studies (Krayner 1931; Krayner and Mendez 1942; Somani and Blum 1966; Takeda et al. 1973; Ishikawa et al. 1978, 1983; Ono et al. 1984a, b; Caffrey et al. 1986; Hausknecht et al. 1986; Fessler et al. 1988; Seifen et al. 1987, 1988; Naka et al. 1989). More recently, the rat model has been preferred (Dietz 1984, 1987; Onwochei et al. 1987, 1988; Kashimoto et al. 1987, 1990, 1994, 1995, Fukuse et al. 1995).

PROCEDURE

Wistar rats weighing 300–320 g are anesthetized with 50 mg/kg pentobarbitone i.p. Tracheotomy is performed and intermittent positive pressure ventilation is instituted with air. The chest is opened and flooded with ice-cold saline and the heart arrested. Cannulae are inserted into the aorta and the superior (for measurement of central venous pressure) and inferior vena cavae. The heart-lung preparation is perfused with a solution containing rat blood cells from another rat and Krebs-Ringer bicarbonate buffer, with hematocrit and pH of 25% and 7.4, respectively. Concentration of the buffer constituents (mM): NaCl 127, KCl 5.1, CaCl₂ 2.2, KH₂PO₄ 1.3, MgSO₄ 2.6, NaHCO₃ 15, glucose 5.5 and heparin. The perfusate pumped from the aorta passes through a pneumatic resistance and is collected in a reservoir maintained at 37°C and then returned to the inferior vena cava. In this model, no other organs except the heart and lung are perfused. Cardiac output is determined by the inflow as long as the heart does not fail. Mean arterial pressure is regulated by the pneumatic resistance. Heart rate is recorded by a bioelectric amplifier and cardiac output is measured with an electromagnetic blood flow meter. Arterial pressure and right atrial pressure are measured with transducers and amplifiers. The heart is perfused initially with cardiac output of 30 ml/min and mean arterial pressure of 80 mm Hg. Test drugs are administered into the perfusate 5 min after start of the experiment.

EVALUATION

Hemodynamic data within groups are analyzed by two-way analysis of variance (ANOVA) with repeated measures. Recovery time is measured by the Kruskal-Wallis test. The other data are analyzed by one-way ANOVA followed by the Dunnett test for multiple comparisons.

MODIFICATIONS OF THE METHOD

Using the Starling heart-lung preparation in dogs, Woltenberger (1947) studied the energy-rich phosphate supply of the failing heart.

Shigei and Hashimoto (1960) studied the mechanism of the heart failure induced by pentobarbital, quinine, fluoroacetate and dinitrophenol in dog's heart-lung preparation and effects of sympathomimetic amines and ouabain on it.

Imai et al. (1961) used heart-lung preparations of the dog to study the cardiac actions of methoxamine with special reference to its antagonistic action to epinephrine.

Capri and Oliverio (1965), Beaconsfield et al. (1974) used the heart-lung preparation of the **guinea pig**.

Robicsek et al. (1985) studied the metabolism and function of an autoperfused heart-lung preparation of the **dog**.

The **dog heart-lung preparation** was used by Seifen et al. (1988) to study the interaction of a calcium channel agonist with the effects of digoxin, by Somani and Blum (1966) to study blockade of epinephrine- and ouabain-induced cardiac arrhythmias in the dog,

by Riveron et al. (1988) to investigate the energy expenditure of an autoperfusing heart-lung preparation, by Namakura et al. (1987) to study the role of pulmonary innervation in an *in situ* lung-perfusion preparation as a new model of neurogenic pulmonary edema,

by Hausknecht et al. (1986) to investigate the effects of lung inflation on blood flow during cardiopulmonary resuscitation,

by Caffrey et al. (1986) to evaluate the effect of naloxone on myocardial responses to isoproterenol,

by Ono et al. (1984) to estimate the cardiodepressant potency of various beta-blocking agents,

by Ishikawa et al. (1983) for a graphical analysis of drug effects in the dog heart-lung preparation – with particular reference to the pulmonary circulation and effects of norepinephrine and 5-hydroxytryptamine,

by Iizuka (1983) to study the cardiac effects of acetylcholine and its congeners,

by Fessler et al. (1988) to investigate the mechanism of reduced LV afterload by systolic and diastolic positive pleural pressure, by Takeda et al. (1973) to study the cardiac actions of oxprenolol.

Beaconsfield et al. (1974) used the heart-lung preparation of **guinea pigs** to study the cardiac effect of delta-9-tetrahydrocannabinol.

The **rabbit** autoperfusing heart-lung preparation was used by Muskett et al. (1986, 1988).

The isolated heart-lung preparation in the **cat** was described by Beaufort et al. (1993).

Kontos et al. (1987, 1988) harvested heart-lung blocks from **calves**.

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A.3.1.2

Isolated Heart According to Langendorff

PURPOSE AND RATIONALE

More than 100 years ago Langendorff (1895) described studies on isolated surviving mammalian hearts using mainly cats as donors. Since then, the method has been improved from the technical site and is nowadays used for studies with guinea-pig, rabbit or rat hearts. In principle, the heart is perfused in retrograde direction from the aorta either at constant pressure or at constant flow with oxygenated saline solutions. Retrograde perfusion closes the aortic valves, just as in the *in situ* heart during diastole. The perfusate is displaced through the coronary arteries flowing off the coronary sinus and the opened right atrium. In this original set-up the ventricles do not fill with perfusate and therefore do not perform pressure-volume work. Parameters usually measured are: contractile force, coronary flow and cardiac rhythm.

PROCEDURE

Guinea pigs of either sex weighing 300–500 g are sacrificed by stunning. For studies of biochemical parameters in tissue and perfusate, removal of the heart dur-

ing barbiturate anesthesia and artificial respiration is recommended. The heart is removed as quickly as possible and placed in a dish containing Ringer's solution at 37°C. Associated pericardial and lung tissue are removed. The aorta is located and cut just below the point of its division. A glass or plastic cannula is introduced into the aorta, tied with two threads and perfusion is started with oxygenated Ringer's solution or Krebs-Henseleit buffer. The heart is transferred to a double walled plexiglass perfusion apparatus which is kept at 37°C by the water from a thermostat. Oxygenated Ringer's solution is perfused at a constant pressure of 40 mm Hg and at a temperature of 37°C from a reservoir. A small steel hook with a string is attached to the apex of the heart. Contractile force is measured isometrically by a force transducer with a preload of 2.5 g and recorded on a polygraph. Coronary flow is measured by a drop counter. Alternatively, flow measurements can be performed using a mechanic-electronic flow meter consisting of a vertical pipe and a magnetic valve (Hugo Sachs Electronic KG, Germany). Heart rate is measured through a chronometer coupled to the polygraph. Drugs are injected into the perfusion medium just above the aortic cannula.

CRITICAL ASSESSMENT OF THE METHOD

A reappraisal of the LANGENDORFF heart preparation was given by Broadley (1979) underlining the usefulness to test coronary vasodilating drugs. The value of the LANGENDORFF method can be best assessed by demonstrating a few of its applications in physiology and pharmacology. Direct effects can be measured as well as the antagonism against various physiological and pharmacological agents.

MODIFICATIONS OF THE METHOD

A survey on various modifications of the LANGENDORFF-technique and the **isolated working heart preparation** has been given by Ross (1972).

Neely et al. (1967) inserted a second cannula into a pulmonary vein or the left atrium. Perfusate from a reservoir flows via this cannula through the mitral valve into the left ventricle. During the systole of the heart, the left ventricle repumps the perfusate through the aorta into the reservoir. The perfusate flowing through the coronary arteries and dripping off from the outside of the heart is collected in a vessel below the heart and recirculated into the reservoir with a roller pump.

Flynn et al. (1978) underlined the difference of this working heart preparation to the original LAN-

GENDORFF method and reported the effects of histamine and noradrenaline on peak left ventricular systolic pressure, contractility, sinus rate, coronary flow, aortic flow, total cardiac output, and external pressure-volume work. Therefore, this method is reported separately.

Ishii et al. (1996) measured simultaneously Ca^{2+} -dependent indo-1 fluorescence and left ventricular pressure on a beat-to-beat basis in Langendorff guinea pig hearts and investigated the changes in Ca^{2+} transient and left ventricular function during positive inotropic stimulation and myocardial ischemia.

Hukovic and Muscholl (1962) described the preparation of the isolated **rabbit** heart with intact sympathetic nervous supply from the right stellate ganglion.

Hendrikx et al. (1994) used the isolated perfused rabbit heart to test the effects of an Na^+/H^+ exchange inhibitor on postischemic function, resynthesis of high-energy phosphate and reduction of Ca^{2+} overload.

Michio et al. (1985) modified the Langendorff method in rabbits to a working heart preparation by cannulating the left atrium. At a pressure of 20 cm H_2O in the left atrium, the heart pumped the solution against a hydrostatic pressure of 100 cm H_2O . Aortic flow, systolic aortic pressure, coronary flow, and heart rate were measured.

The influence of an ACE-inhibitor on heart rate, lactate in the coronary effluente and GTP-level in the myocardium after 60-min hypothermic cardiac arrest was studied in working heart preparation of rabbits by Zegner et al. (1996).

Gottlieb and Magnus (1904) introduced the so called “**balloon method**”. A small balloon fixed to the tip of a catheter is filled with water and inserted into the left ventricle via one of the pulmonary veins, the left atrium and the mitral valve. The balloon size has to fit the volume of the left ventricle and therefore its size depends on the animal species and body weight. The catheter can be fixed by tying the pulmonary vein stems. Via a three-way valve, the balloon can be extended to a given preload. The beating heart now exerts a rhythmic force to the balloon and thus to the membrane of a pressure transducer. The advantages of this method are that force development and preload can be stated reproducibly in pressure units [mm Hg], left ventricular contraction curves can be used for further calculations, and continuous heart rate recordings can be carried out without any problems when using a rate meter.

Sakai et al. (1983) reported a similar method adapted to **mice**.

Bardenheuer and Schrader (1983) described a method whereby the balloon is inserted into the left ventricle as described above. However, isovolumetric pressure in the left ventricle is not measured. Instead, the fluid in the balloon is pumped through the cannula into a closed extra corporal circulation. The fluid is forced into one direction by 2 recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K^+ -arrested heart by injection of dental cement (Palavit 55, Kulzer and Co, GmbH, Germany). During each heart beat the fluid volume expelled from the balloon corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated **rat** hearts (Linz et al. 1986; Linz et al. 1990):

- LVP (left ventricular pressure) with Statham pressure transducer P 23 DB, which on differentiation yielded $\text{LV } dp/dt_{\text{max}}$ and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.
- Myocardial oxygen consumption ($M\text{VO}_2$) [ml/min/g wet weight] is calculated according to the equation:

$$M\text{VO}_2 = CF \times (P_a - P_v) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mm Hg), P_v is the oxygen partial pressure of the venous effluent perfusate [mm Hg], and c is the 0.0227 ml O_2 /ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37°C (Zander and Euler 1976).

For the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the perfusate, samples are taken from the coronary effluent.

After the experiments, hearts are rapidly frozen in liquid nitrogen and stored at -80°C . Of the left ventricle, 500 mg are taken, put into 5 ml ice-cold HClO_4 and disrupted with an Ultra-Turrax (Junke and Kunkel, Ika-Werk, Type TP). Glycogen is hydrolyzed with amyloglycosidase (pH 4.8) and determined as glucose. Furthermore, ATP and creatine phosphate are measured.

Avkiran and Curtis (1991) constructed a dual lumen aortic cannula which permits independent perfusion of left and right coronary beds in isolated rat hearts without necessitating the cannulation of individual arteries.

Igic (1996) described a modification of the isolated perfused working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted into the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle, and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses coronary vessels and the rest is expelled. Because the inner cannula can be easily retracted into the outer cannula, which is placed in the aorta, the preparation provides an opportunity to use the same heart as a "working" or "non-working" model for investigating functions of the heart.

By labeling glucose, lactate, or fatty acids in the perfusate with ^3H or ^{14}C , Barr and Lopaschuk (1997) directly measured energy metabolism in the isolated rat heart.

Krzeminski et al. (1991) described a new concept of the isolated heart preparation with on-line computerized data evaluation. Left ventricular pressure was recorded by means of a balloon-catheter, while special suction electrodes obtained the high-amplitude, noise-free electrogram recordings. The coronary effluent partial pressure of oxygen was continuously monitored, which enabled the calculation of myocardial oxygen consumption (MVO_2). The effluent partial pressure of carbon dioxide and pH value were also measured simultaneously. A computerized system of data acquisition, calculation, storage, and end report was described.

Döring and Dehnert (1988) described continuous simultaneous **ultrasonic recording** of two cardiac diameters in an isolated perfused guinea-pig heart. For the measurement of the left ventricular transversal diameter the ultrasonic transmitter was positioned at the epicardium at the largest cardiac diameter. The cor-

responding ultrasonic receiver was inserted through the right atrium into the right ventricle to approximately the same height as the transmitter. In the right ventricle, which is empty in the isolated perfused LANGENDORFF-heart, it was automatically positioned opposite to the transmitter. Additional transducers were placed both at the heart's base and apex for assessment of the ventricular longitudinal diameter.

Several authors used the **isolated perfused mouse heart**.

Bittner et al. (1996) described a work-performing heart preparation for myocardial performance analysis in murine hearts using a modified Langendorff apparatus.

Sumaray and Yellon (1998a, b) constructed a specially designed Langendorff apparatus that allows perfusion of the isolated **mouse** heart. These authors reported that ischemic preconditioning reduces infarct size following global ischemia in the murine myocardium.

Brooks and Apstein (1996) measured left ventricular systolic and diastolic pressures in the isovolumically contracting (balloon in the left ventricle) mouse hearts.

Sutherland et al. (2003) reviewed characteristics and cautions in the use of the isolated perfused heart of mice.

Wang et al. (2001) studied the relationship between ischemic time and ischemia/reperfusion injury in isolated Langendorff-perfused mouse hearts.

Tejero-Taldo et al. (2002) reported that α -adrenergic receptor stimulation produces late preconditioning through inducible nitric oxide synthase in mouse heart.

Ross et al. (2003) found that the $\alpha_{1\text{B}}$ -adrenergic receptor decreases the inotropic response in the mouse Langendorff heart model.

Bratkovsky et al. (2004) measured coronary flow reserve in isolated hearts from mice.

Plumier et al. (1995) generated **transgenic mice** expressing the human heart heat shock protein 70. Upon reperfusion of the hearts after 30 min of ischemia in the Langendorff preparation, transgenic hearts versus non transgenic hearts showed significantly improved recovery of contractile force.

Hannan et al. (2000) compared ENOS knockout and wild-type mouse hearts which were perfused in a Langendorff apparatus with Krebs bicarbonate buffer and subjected to 20 min of global normothermic ischemia followed by 30 min of reperfusion. Myocardial function was measured using a ventricular balloon to determine time to onset of contraction, left ventricular

developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and heart rate-pressure product (RPP).

Sheikh et al. (2001) generated transgenic mice over-expressing fibroblast growth factor (FGF)-2 protein in the heart. An isolated mouse heart model of ischemia-reperfusion injury was used to assess the potential of endogenous FGF-2 for cardioprotection.

APPLICATIONS

Positive Inotropic Effects

While negative inotropic substances can be tested in a heart beating with normal force, the evaluation of a positive inotropic compound usually requires that cardiac force is first reduced. Acute experimental heart failure can be induced by an overdose of barbiturates, such as sodium thiopental, or calcium antagonists. This kind of cardiac failure can be reversed by β -sympathomimetic drugs, cardiac glycosides, or increased Ca^{+2} concentration. In this way, the potential β -sympathomimetic activity of a new drug can be measured using isoproterenol as standard. After thiopental-Na treatment, left ventricular pressure (LVP) and dp/dt_{max} decrease considerably, whereas coronary flow is slightly enhanced. β -Sympathomimetic drugs restore LVP and dp/dt_{max} and keep coronary blood flow elevated.

Cardiac glycosides increase LVP and dp/dt_{max} and leave coronary flow unchanged.

Negative Inotropic Effects

The effects of a β -sympathomimetic drug such as isoproterenol at doses of 0.05 to 0.2 μg increasing contractile force as well as heart frequency are registered. After injection of a β -blocker, the effects of isoproterenol are attenuated. The effects of a potential β -blocking agent can be tested comparing the isoproterenol inhibition versus a standard such as propranolol (0.1 mg).

Coronary Vessel Dilating Effect

The LANGENDORFF heart has been extensively used for assessing the coronary dilating activity of drugs (Broadley 1979). Rothaul and Broadley (1982) demonstrated the release of coronary vasodilator mediators from guinea pig isolated hearts by a technique employing donor and recipient hearts in series.

Calcium-Antagonism

In order to demonstrate the effect of calcium-antagonists, 1 to 5 mg BaCl_2 are injected which induce a pronounced spasm of the coronary arteries thereby reduc-

ing the coronary flow. Five min later, the test drug is injected. Active compounds have a relaxing effect on coronary arteries indicated by an increase of coronary flow. After this effect has waned, BaCl_2 is injected again and the test drug or a standard drug, e. g. nifedipine, is tested. The increase of coronary flow is expressed as percentage of flow during BaCl_2 spasm and compared with the effect of the standard. Using various doses, dose-response curves can be established.

Effect on Potassium Outflow Induced by Cardiac Glycosides

Lindner and Hajdu (1968) described a method using the LANGENDORFF heart in which contractile force, coronary flow, and the potassium content in the coronary outflow was determined by flame photometry. Increase in potassium outflow correlates well with the positive inotropic effect.

Gradual Determination of Hypoxic Damage

Lindner and Grötsch (1973) measured the enzymes creatine phosphokinase (CPK), lactate dehydrogenase (LDH), α -hydroxybutyrate dehydrogenase (α -HBDH), and glutamicoxalacetic transaminase (GOT) in the effluent of a guinea pig heart preparation under varying degrees of hypoxia. Potassium content and oxygen tension in the inflowing and outflowing solution were determined. The heart rate, the amplitude of contraction and the rate of coronary vessel perfusion were recorded additionally.

Metabolic Studies with Nuclear Magnetic Resonance

Using ^{31}P , studies on metabolism of nucleotides and phosphorylated intermediates of carbohydrates in isolated hearts have been performed (Garlick et al. 1977; Jacobus et al. 1977; Hollis et al. 1978; Matthews and Radda 1984).

Arrhythmogenic, Anti-Arrhythmic and Antifibrillatory Effects

The LANGENDORFF heart preparation is also used to test the influence of compounds on cardiac rhythm. For recording monophasic action potentials, suction electrodes are applied on the heart. Ventricular fibrillation can be induced by simultaneous injection of digitoxin (12.5–25.0 μg) and aconitine (12.5–25.0 μg) into the perfusion fluid (Lindner 1963). Cardiac glycosides shorten the refractory period, decrease the conduction velocity and increase heterotopic stimulus generation. Aconitine increases markedly heterotopic stimulus generation. Both compounds together induce invariably ventricular fibrillation. Anti-arrhythmic com-

pounds can be tested in this way. Fibrillation is inhibited, at least partially, by 20 µg prenylamine, 10–20 µg quinidine or 20 µg ajmaline.

Takeo et al. (1992) described protective effects of anti-arrhythmic agents on oxygen-deficiency-induced contractile dysfunction of isolated perfused hearts. Hypoxia in isolated rabbit hearts was induced by perfusing the heart for 20 min with Krebs-Henseleit buffer saturated with a gas mixture of 95% N₂ and 5% CO₂ containing 11 mM mannitol. After hypoxic perfusion, the heart was reoxygenated for 45 min with oxygenated buffer containing glucose.

Dhein et al. (1989) studied the pathway and time course of the epicardial electrical activation process by means of a computer-assisted epicardial potential mapping, using a matrix of 256 unipolar AgCl electrodes (1 mm spatial and 0.25 ms temporal resolution) in isolated rabbit hearts perfused according to the Langendorff technique. From the activation times of the surrounding electrodes, the direction and velocity of activation for each electrode were calculated, thereby allowing construction of an epicardial vector field. The method was used for the assessment of arrhythmogenic and anti-arrhythmic drug activity.

Electrical Stimulation and Antifibrillatory Effect

Ventricular fibrillation can be induced in the LANGENDORFF preparation by reducing the glucose content of the perfusion medium to 0.25 g/1000 ml and the KCl content to 0.12 g/1000 ml. (Burn et al. 1957, 1960; Lindner 1963). After a perfusion period of 20 min, 10 µg epinephrine are injected into the perfusion cannula. Immediately afterwards, the heart is stimulated with a current of 40 Hz and 5 mA for 2 min. This procedure is repeated every 10 min. Standard conditions are achieved when the fibrillation continues without further electrical stimulation. Hearts treated in this way serve as controls. Other hearts stimulated in the same way are treated with continuous infusion of the test drug or the standard via the perfusion medium. Differences in the incidence of fibrillations are calculated using the χ^2 test.

Electrophysiological Evaluation of Cardiovascular Agents

Balderston et al. (1991) modified the Langendorff technique in rabbit hearts in order to perform electrophysiologic studies. His bundle electrograms were measured with a plunge electrode and allowed atrioventricular nodal physiology to be evaluated directly. Atrial conduction and refractoriness, atrioventricular node conduction and refractoriness, His-Purkinje conduction, and ventricular conduction and refractoriness

could be accurately measured. The effects of verapamil and flecainide were described.

EDRF Release from the Coronary Vascular Bed

Lamontagne et al. (1992) isolated platelets from blood of healthy human donors and injected platelets boluses into the perfusion line of the Langendorff preparation of a rabbit heart. In the effluent cyclic GMP was determined as an index for EDRF release.

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A.3.1.3

Coronary Artery Ligation in Isolated Working Rat Heart

PURPOSE AND RATIONALE

In working heart preparations of rats, ischemia can be induced by clamping the left coronary artery close to its origin. After removal of the clip, changes in the reperfusion period can be observed. Prevention of these symptoms can be an indicator of the efficacy of coronary drugs.

PROCEDURE

The preparation used is a modification of an isolated working heart preparation originally used for guinea pig hearts (Bardenheuer and Schrader 1983). Wistar rats of either sex weighing 280–300 g are sacrificed by decapitation. The hearts are removed and dissected free from the epicard and surrounding connective tissue. A cannula is introduced into the aorta from where the coronary vessels are perfused with the non-recirculated perfusion medium according to the Langendorff technique. In the left ventricle a balloon closely fitting the ventricular cavity is placed and connected to an artificial systemic circulation. The fluid in the balloon is pumped through a cannula into the closed extra corporal circulation being forced into one direction by 2 recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K⁺-arrested heart by injection of dental cement (Palavit 55, Kulzer and Co, GmbH, Germany). During each heart beat the fluid volume pressed from the balloon, corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated rat hearts (Linz et al. 1986):

LVP (left ventricular pressure) with Statham pressure transducer P23 DB, which on differentiation yielded LV dp/dt_{\max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO₂ is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH

73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.

Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation:

$$MVO_2 = CF \times (P_a - P_v) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mm Hg), P_v is the oxygen partial pressure of the venous effluent perfusate [mm Hg], and c is the 0.0227 ml O₂/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37°C (Zander and Euler 1976).

Coronary Artery Ligation

For coronary artery occlusion experiment (Scholz et al. 1992, 1993), the isolated working hearts are perfused for a period of 20 min (pre-ischemic period) with modified Krebs-Henseleit buffer at a constant pressure of 65 mm Hg. Thereafter, acute myocardial ischemia is produced by clamping the left coronary artery close to its origin for 15 min (ischemic period). The clip is then reopened, and changes during reperfusion are monitored for 30 min (reperfusion period). After coronary artery ligation and reperfusion the hearts develop ventricular fibrillation.

From the coronary effluent samples are taken for lactate, lactate dehydrogenase (LDH), and creatine kinase (CK) determinations. After the experiment, glycogen, lactate, ATP, and creatine phosphate in myocardial tissue are measured.

The test drugs are given into the perfusion medium either before occlusion or 5 min before reperfusion. For *ex vivo* studies, the rats are treated orally with the test drug 1 h before sacrifice and preparation of the isolated working heart.

EVALUATION

The incidence and duration of ventricular fibrillation after treatment with coronary drugs is compared with controls. Left ventricular pressure, LV dP/dt max, and coronary flow are reduced after coronary constriction by angiotensin II, whereas enzyme activities in the effluent are increased and the myocardial content of glycogen, ATP and creatine phosphate are decreased. Cardiac protective drugs have the opposite effects. The values of each parameter are statistically compared with controls.

MODIFICATIONS OF THE METHOD

Vogel and Lucchesi (1980) described an isolated, blood perfused, **feline** heart preparation for evaluating pharmacological interventions during myocardial ischemia. Ventricular function was measured with a fluid-filled latex balloon within the left ventricle.

Vleeming et al. (1989) ligated the left coronary artery in rats after thoracotomy in ether anesthesia. Forty-eight hours after the operation, the hearts were prepared for retrograde constant pressure perfusion, according to the Langendorff technique.

Igic (1996) presented a new method for the isolated working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted in the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle, and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses the coronary vessels and the rest is expelled. Because the inner cannula can easily be retracted into the outer cannula, which is placed in the aorta, this preparation provides an opportunity to use the same heart as a "working" or "non-working" model for investigating functions of the heart.

Pepe and McLennan (1993) described a maintained afterload model of ischemia in erythrocyte-perfused isolated working hearts of rats.

Further characterization of the pathophysiological reactions of the isolated working heart was performed by Linz et al. (1999). The external heart power (EHP) [mJ/min/g] was calculated using the formula:

$$\begin{aligned} EHP_{LV} &= \text{pressure} - \text{volume} + \text{acceleration work} \\ &= [SV(MAP - LAP)] \\ &\quad + [1/2SV \times d \times (SV/\pi r^2 e^2)] HR g_{LVwwt}^{-1} \end{aligned}$$

SV indicates stroke volume; *MAP*, mean aortic pressure; *LAP*, mean left arterial pressure; *d*, specific weight perfusate (1.004 g/cm³); *r*, inner radius of aortic cannula; *e*, ejection time; *HR*, heart rate; *LV*, left ventricle; *LVwwt*, left ventricular wet weight.

The function of the left ventricle was altered by changing the aortic pressure (afterload) at constant left atrial filling load (preload). By adjusting the Starling resistance, the aortic outflow could be switched during 1 min from the fixed baseline afterload to a preset higher afterload producing step-wise rises in mean arterial pressure.

Lee et al. (1988) studied the effects of acute global ischemia on cytosolic calcium transients in perfused isolated **rabbit** hearts with the fluorescent calcium indicator indo 1. Indo 1-loaded hearts were illuminated at 360 nm, and fluorescence was recorded simultaneously at 400 and 550 nm from the epicardial surface of the left ventricle. The F_{400}/F_{550} ratio was calculated by an analog circuit, which allowed cancellation of optical motion artifact. The resulting calcium transients were registered simultaneously with the ventricular pressure and demonstrated a rapid upstroke and slow decay similar to those recorded in isolated ventricular myocytes. Global ischemia rapidly suppressed contraction, but it produced a concurrent increase in the systolic and diastolic levels of calcium transients, together with an increase in the duration of the peak.

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A.3.1.4

Isolated Working Heart Model in Infarcted Rat Heart

PURPOSE AND RATIONALE

The model of chronic heart failure in spontaneously hypertensive rats described by Itter et al. (2004a) (see A.6.0.1.2) has been used by the same group to study the isolated working heart in rats after chronic infarction (Itter et al. 2004b).

PROCEDURE

Animals and Methods

WKY/NHsd and SHR/NHsd rats at an age of 4 months were randomized into two groups – sham and myocardial infarction (MI). The sham procedure consisted of opening the pericardium and placing a superficial suture in the epicardium of the left ventricle (LV). Chronic heart failure (CHF) was induced by permanent (8 weeks) occlusion of the left coronary artery 2 mm distal to the origin from the aorta resulting in a large infarction of the free left ventricular wall.

Eight weeks after surgery, parameters indicating CHF were measured. Cardiac hypertrophy, function and geometric properties were determined by the “working heart” mode and *in vivo* determinations by MRI and heart weight.

Surgery

The rats were anesthetized with a mixture of ketamine/xylazine (35/2 mg/kg i.p.). The left ventrolateral thorax was shaved and prepared to create a disinfected surgical access area. When stable anesthesia was achieved the animals were placed on a small animal operation table, intubated and ventilated with

room air using a small animal ventilator (KTR-4, Hugo Sachs Elektronik, March-Hugstedten, Germany). The level of anesthesia was deemed as adequate following loss of the pedal withdrawal reflex and absence of the palpebral reflex. Reflexes were evaluated before surgery. The operation took 5 min. The tidal volume was adjusted at 3–5 ml and the ventilation rate was 40 breaths/min. Left thoracotomy was performed via the third intercostal space. The heart was exposed and the pericardium opened. The left main coronary artery was ligated with Perma-Hand silk 4–0 USP (Ethicon, Nordersredt, Germany) near its origin at the aorta (2 mm distal to the edge of the left atrium). Ligation resulted in infarction of the free left ventricular wall. Ligation was deemed successful when the anterior wall of the left ventricle turned pale. At this point the lungs were hyperinflated by increasing the positive end-expiratory pressure, and the chest was closed. The rats were placed on a heating pad and covered with a layer of unbleached tissue paper. The rats were extubated following return of reflexes. They were continuously monitored until they started moving in their cages. To avoid ventricular arrhythmias, lidocaine (2 mg/kg i.m.) was given before surgery. To prevent acute lung edema, the rats received furosemide (Lasix, 2 mg/kg body weight twice daily for 3 days) via the drinking water. To avoid pain and distress the rats received metamizol treatment (Novalgin, 0.1 mg/kg body weight i.m.) once, directly after the recovery period.

Before killing the animals 8 weeks after MI, non-invasive sequential nuclear magnetic resonance (NMR) measurements of heart geometric properties were done. Thereafter the animals were anesthetized with pentobarbitone (180 mg/kg i.p. Pentobarbital) and subsequently heparinized (Heparin Natrium 500 I.U./100 g body weight i.p.). Once stable anesthesia was achieved (stage III 3, reflexes absent), the animals were connected to an artificial respirator via a PE (polyethylene) tube inserted into the trachea and ventilated with room air. A transverse laparotomy and a right anterolateral thoracotomy were performed, and the heart was rapidly removed for the evaluation of its function in the working heart mode. The heart was immersed in physiological buffer chilled to 4°C. The aorta was dissected free and mounted onto a cannula (internal diameter: 1.4 mm) attached to perfusion apparatus. The hearts were perfused according to the method of Langendorff with an oxygenated (95% O_2 /5% CO_2) non-circulating Krebs–Henseleit solution of the following compositions (mM): NaCl, 118; KCl, 4.7; CaCl_2 , 2.52; MgSO_4 , 1.64; NaHCO_3 ,

24.88; KH_2PO_4 , 1.18; glucose, 5.55; and Na pyruvate, 2.0 at a perfusion pressure of 60 mmHg. Any connective tissue, thymus or lung was carefully removed. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for the determination of coronary flow and venous $p\text{O}_2$ measurements. The left atrium was cannulated via an incision of the left auricle. All pulmonary veins were ligated close to the surface of the atria.

When a tight seal with no leaks had been established and after a 15-min equilibration period, the hearts were switched into the working mode, using a filling pressure (preload) of 12 mmHg in WKY/NHsd and 18 mmHg in SH rats. The afterload pressure was 60 mmHg in WKY/NHsd and 80 mmHg in SH rats. After validation of the basis parameters the afterload pressure was enhanced in a cumulative manner from an additional 20 mmHg to 140 mmHg. Thereafter the isovolumetric maxima were determined by enhancing the preload pressure in steps of 5 mmHg to 30 mmHg.

Flow and pressure signals for computation were obtained from the PLUGSYS-measuring system (Hugo Sachs Elektronik, March-Hugstedten, Germany). Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 s, using the software Aquire Plus V1.21f (PO-NE-MAH, Hugo Sachs Elektronik, March-Hugstedten, Germany).

Determination of Infarct Size

After the evaluation of the external heart work, the total heart weight, and the left and right ventricular weights were determined. The left ventricle was then sectioned transversely into four slices from the apex to the base. Eight pictures were taken of each rat heart, two from each slice. Total infarct size was determined by planimetry of the projected and magnified slices. The areas of infarcted tissue as well as the intact myocardium of each slice were added together and averaged. The infarcted fraction of the left ventricle was calculated from these measurements and expressed as a percentage of the left ventricular mass. The left ventricular perimeter, diameter, infarct scar length, as well as wall thickness and infarct wall thinning were determined as well.

EVALUATION

The data are given as mean \pm SEM. Statistics were performed using the SAS system statistics package (SAS Institute, Cary, N.C., USA) with a sequential rejection *t*-test according to Holm (1979).

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A.3.1.5

Relaxation of Bovine Coronary Artery

PURPOSE AND RATIONALE

Eicosanoids can regulate the tonus of coronary arteries. Prostacyclin induces relaxation, whereas thromboxane A_2 causes contraction. Spiral strips from bovine coronary artery can be used for assaying relaxation activity of test compounds (Dusting et al. 1977)

PROCEDURE

Freshly slaughtered beef hearts are immersed in cold oxygenated Krebs solution and immediately transported in a thermos flask to the laboratory. The left descending coronary artery and several of its primary branches are cut into spiral strips (about 20 mm long and 2–3 mm wide). The specimens can be stored up to 48 h at 4°C. The artery strips are suspended in a 4 ml organ bath under an initial tension of 2 g and immersed in a Krebs' bicarbonate solution at 37°C being gassed with oxygen containing 5% CO_2 throughout the experiment. The Krebs solution contains a mixture of antagonists to inhibit any actions from endogenous acetylcholine, 5-hydroxytryptamine, histamine or catecholamines (hyoscine hydrobromide 10^{-7} g/ml, methysergide maleate 2×10^{-7} g/ml, mepyramine maleate 10^{-7} g/ml, propranolol hydrochloride 2×10^{-6} g/ml). The strips are superfused with a solution of the test compounds in concentrations of 0.01, 0.1, 1.0 $\mu\text{g}/\text{ml}$ at a rate of 10–20 ml/min with oxygenated Krebs solution containing the mixture of antagonists. Isometric contractions are recorded with Grass force-displacement transducers (type FT 03 C) on a Grass polygraph. The strips are superfused with Krebs' solution 3 h prior to the experiment. Standard compounds are 100 ng/ml PGE_2 inducing contraction and 100 ng/ml PGI_2 inducing pronounced relaxation.

EVALUATION

The relaxation induced by the test compound is expressed as percentage of maximal response to 100 ng/ml PGI_2 .

MODIFICATIONS OF THE METHOD

Campell and Paul (1993) measured the effects of diltiazem on isometric force generation, $[Ca^{2+}]_i$, and energy metabolism in the isolated **porcine** coronary artery.

Li et al. (1997) determined the ability of analogues of human α -calcitonin gene-related peptide to relax isolated porcine coronary arteries precontracted with 20 mM KCl.

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A.3.2

In Vivo Methods

A.3.2.1

Isoproterenol Induced Myocardial Necrosis in Rats

PURPOSE AND RATIONALE

Cardiac necrosis can be produced by injection of natural and synthetic sympathomimetics in high doses. Infarct-like myocardial lesions in the rat by isoproterenol have been described by Rona et al. (1959). These lesions can be totally or partially prevented by several drugs such as sympatholytics or calcium-antagonists.

PROCEDURE

Groups of 10 male Wistar rats weighing 150–200 g are pretreated with the test drug or the standard either s.c. or orally for 1 week. Then, they receive 5.25 and 8.5 mg/kg isoproterenol s.c. on two consecutive days. Symptoms and mortality in each group are recorded and compared with those of rats given isoproterenol alone. Forty-eight hours after the first isoproterenol administration, the rats are sacrificed and autopsied. The hearts are removed and weighed, and frontal sections are embedded for histological examination.

EVALUATION

Microscopic examination allows the following grading:

Grade 0: no change

Grade 1: focal interstitial response

Grade 2: focal lesions in many sections, consisting of mottled staining and fragmentation of muscle fibres

Grade 3: confluent retrogressive lesions with hyaline necrosis and fragmentation of muscle fibres and sequestrating mucoid edema

Grade 4: massive infarct with occasionally acute aneurysm and mural thrombi

For each group the main grade is calculated with the standard deviation to reveal significant differences.

CRITICAL ASSESSMENT OF THE METHOD

The test has been used by many authors for evaluation of coronary active drugs, such as calcium-antagonists and other cardioprotective drugs like nitroglycerin and molsidomine (Vértesy et al. 1991; Classen et al. 1993).

MODIFICATIONS OF THE METHOD

Yang et al. (1996) reported a protective effect of human adrenomedullin^{13–52}, a C-terminal fragment of adrenomedullin^{10–52} on the myocardial injury produced by subcutaneous injection of isoproterenol into rats.

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A.3.2.2

Myocardial Infarction After Coronary Ligation in Rodents

PURPOSE AND RATIONALE

Ligation of the left coronary artery in rats as described by Selye (1960) induces an acute reduction in pump function and a dilatation of left ventricular chamber. The method has been used to evaluate beneficial effects of drugs after acute (Chiariello et al. 1980; Flaim and Zelis 1981; Bernauer 1985) or chronic (Innes and Weisman 1981; Pfeiffer et al. 1985; Linz et al. 1996) treatment.

PROCEDURE

Male Sprague Dawley rats weighing 200–300 g are anesthetized with diethylether. The chest is opened by a left thoracotomy, and a thread is inserted near the middle of the lateral margin of the cutaneous wound and carried through a tunnel of the left pectoral muscle around the cranial half of the incision. The heart is gently exteriorized by pressure on the abdomen. A ligature is placed around the left coronary artery, near its origin, and is tightened. Within seconds, the heart is repositioned in the thoracic cavity, and the ends of the musculocutaneous thread are tightened to close the chest wall and enable the animal to breathe spontaneously.

The speed of the procedure renders mechanical respiration unnecessary.

To evaluate drug effects, the rats are treated 5 min after and 24 h after occlusion by subcutaneous injection (standard 5 mg/kg propranolol).

Two days after surgery, the rats are anesthetized with 60 mg/kg i.p. pentobarbital and the right carotid artery is cannulated with a polyethylene catheter connected to a pressure transducer. The fluid-filled catheter is then advanced into the left ventricle through the aortic valve for measurement of left ventricular systolic and end-diastolic pressure.

After hemodynamic measurements, the heart is arrested by injecting 2 ml of 2.5 M potassium chloride. The chest is opened, and the hearts are isolated and rinsed with 300 mM KCl to maintain a complete diastole. A double-lumen catheter is advanced into the left ventricle through the ascending aorta, the right and left atria are tied off with a ligature, and the right ventricle is opened. The left ventricular chamber is filled with a cryostatic freeze medium through the smaller of the two catheter lumens and connected to a hydrostatic pressure reservoir maintained at a level corresponding to the end-diastolic pressure measured *in vivo*. The outlet (larger lumen) is then raised to the same level as the inlet to allow fluid in the two lumens to equilibrate. The heart is rapidly frozen with hexane and dry-ice.

The hearts are serially cut with a cryostat into 40- μ m-thick transverse sections perpendicularly to the longitudinal axis from apex to base. At a fixed distance, eight sections are obtained from each heart and collected on gelatin-coated glass slides. Sections are air-dried and incubated at 25°C for 30 min with 490 μ M nitroblue tetrazolium and 50 mM succinic acid in 0.2 M phosphate buffer (pH 7.6), rinsed in cold distilled water, dehydrated in 95% ethyl alcohol, cleared in xylene, and mounted with a synthetic resin medium. Viable tissue appears dark blue, contrasting with the unstained necrotic tissue.

EVALUATION

The infarct size can be determined by planimetry and expressed as percentage of left ventricular area, and thickness can be expressed as percentage of non-infarcted ventricular wall thickness (MacLean et al. 1978; Chiariello 1980; Roberts et al. 1983). An automatic method for morphometric analysis with image acquisition and computer processing was described by Porzio et al. (1995).

CRITICAL ASSESSMENT OF THE METHOD

Myocardial infarction following coronary artery ligation in Sprague-Dawley rats is a widely used rat model of heart failure. If the left coronary artery is not completely ligated, heart failure may occur as a consequence of chronic myocardial ischemia (Kajstura et al. 1994).

MODIFICATIONS OF THE METHOD

Johns and Olson (1954) described the coronary artery patterns for mouse, rat, hamster and guinea pig.

Kaufman et al. (1959), Fishbein et al. (1978, 1980) used various histochemical methods for identification and quantification of border zones during the evolution of myocardial infarction.

Sakai et al. (1981) described an **experimental model of angina pectoris in the intact anesthetized rat**. In anesthetized rats the tip of a special carotid cannula was placed closely to the right and left coronary ostium. Single intra-aortic injections of methacholine or acetylcholine (in the presence of physostigmine) developed a reproducible elevation of the ST segment and the T wave of the electrocardiogram. Coronary drugs were tested to prevent these changes.

Ytrehus et al. (1994) analyzed the effects of anesthesia, perfusate, risk zone, and method of infarct sizing in rat and rabbit heart infarction.

Leprán et al. (1981) placed a loose ligature of atraumatic silk around the left anterior descending coronary artery under ether anesthesia in **rats**. Ten days later, acute myocardial infarction was produced by tightening the ligature.

Kouchi et al. (2000) found an increase in $G_{i\alpha}$ protein accompanying progression of post-infarction remodeling in hypertensive cardiomyopathy in **rats**. G protein α subunits were studied with immunoblotting techniques (Böhm et al. 1990). The polyclonal antiserum MB1 was raised in rabbits against the carboxyl-terminal decapeptide of retinal transduction (KENLKDCGLF) coupled to keyhole limpet hemocyanine. The MB1 recognized $G_{i\alpha 1}$ and $G_{i\alpha 2}$ but not $G_{0\alpha}$ and $G_{i\alpha 3}$ (Böhm et al. 1994). The membrane fractions were electrophoresed in SDS-polyacrylamide gels and were transferred to nitrocellulose filters. The filters were incubated with the first antibodies for $G_{i\alpha}$ (MB1) or $G_{s\alpha}$ (RM/1) and then with the second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Amersham). Immunoreactive signals were detected by means of the ECL kit (Amersham).

Liu et al. (1997) found that ligation of the left descending coronary artery in Lewis inbred **rats** pro-

duces an uniformly large infarct with low mortality. The model may be superior to the usual model in Sprague-Dawley rats with a marked variability in infarct size and cardiac dysfunction.

Coronary artery ligation induces left ventricular remodeling with cardiomyocyte apoptosis, myocardial fibrosis indicated by morphological studies and by collagen accumulation, which can be prevented by drug treatment (Yang et al. 1992; Belichard et al. 1994; Nguyen et al. 1998; Sia et al. 2002; Bäcklund et al. 2004).

The naturally occurring peptide ***N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP)** is a inhibitor of pluripotent hematopoietic stem cell proliferation and is normally present in human plasma and circulating mononuclear cells. It is cleaved to an inactive form by the NH_2 -terminal catalytic domain of ACE (Azizi et al. 1996). Acute angiotensin converting enzyme inhibition increases the plasma level of *N*-acetyl-seryl-aspartyl-lysyl-proline (Azizi et al. 1996). By morphological studies and collagen determinations, Rasoul et al. (2004) found an antifibrotic effect of Ac-SDKP and angiotensin converting enzyme inhibition in hypertension in rats. Similarly, Yang et al. (2004) found that Ac-SDKP reverses inflammation and fibrosis in rats with heart failure after myocardial infarction.

Moreover, Azizi et al. (1997) and Le Meur et al. (1998) discussed whether the plasma Ac-SDKP level is a reliable marker of chronic angiotensin converting enzyme inhibition in hypertensive patients. An Ac-SDKP EIA Kit is available from Cayman, Ann Arbor, Mich., USA.

Chen et al. (2004) found inhibition and reversal of myocardial infarction-induced hypertrophy and heart failure by NHE-1 inhibition.

Johns and Olson (1954) described a method of experimental **myocardial infarction by coronary occlusion in small animals**, such as **mouse, hamster, rat and guinea pig**.

Scholz et al. (1995) described a dose-dependent reduction of myocardial infarct size in **rabbits** by a selective sodium-hydrogen exchange subtype 1 inhibitor.

Gomoll and Lekich (1990) tested the **ferret** for a myocardial ischemia/salvage model. Varying combinations of duration of left anterior descending coronary occlusion and reperfusion were evaluated.

Coronary artery ligation in mice

Michael et al. (1995, 1999), and Gould et al. (2001) described the surgical procedure to induce myocardial ischemia in **mice** by ligation of the left anterior descending branch of the left coronary artery.

Infarct and Reperfusion Model

Male C57BL/6 mice 12–16 weeks of age (22.5–30.5 g body weight) were used. Anesthesia was produced by an intraperitoneal injection of pentobarbital sodium (4 mg/ml; 10 μ l/g body weight). Mice were placed in a supine position with paws taped to the operating table. With direct visualization of the trachea, an endotracheal tube was inserted and connected to a Harvard rodent volume-cycled ventilator cycling at 100/min with volume sufficient to adequately expand the lungs but not overexpand. The inflow valve was supplied with 100% oxygen.

For studies of the myocardial response to permanent occlusion, ligation of the anterior descending branch of the left coronary artery was achieved by tying an 8–0 silk suture around the artery. The suture was passed under the artery at a position \sim 1 mm from the tip of the normally positioned left auricle.

For studies of the effect of reperfusion after coronary artery occlusion, the ligature was tied at the same location on the coronary artery used for the permanent occlusion. However, to allow subsequent reestablishment of blood flow, occlusion was produced by placing a 1-mm length of polyethylene (PE) tubing (OD=0.61 mm) on the artery and fixing it in place with the ligature. The artery was then compressed by tightening the ligature, producing myocardial blanching and electrocardiographic (ECG) S-T segment elevation as observed in permanent ligations. After occlusion for the desired time, blood flow was restored by removing the ligature and PE tubing. The chest wall was then closed by a 6–0 Ticron suture with one layer through the chest wall and muscle and a second layer through the skin and subcutaneous layer.

After surgical closing of the chest, the endotracheal tube was removed, warmth was provided by a heat lamp, and 100% oxygen was provided via a nasal cone. The animal was given 0.1 mg/kg butorphanol tartrate as an analgesic, and it became sternally recumbent within 1 h. After surviving the experimental infarct the mice recovered, and this allowed postoperative physiological measurement. Sham-operated mice underwent an identical procedure with placement of the ligature but did not undergo coronary artery occlusion.

MODIFICATIONS OF THE METHOD

Guo et al. (1998) demonstrated the effects of an early and a late phase of ischemic preconditioning in mice. The results demonstrated that, in the mouse, a robust infarct-sparing effect occurred during both the early and the late phases of ischemic preconditioning, although the early phase was more powerful.

Guo et al. (2005) found that late preconditioning induced by NO donors, adenosine A₁ receptor agonists, and δ_1 -opioid receptor agonists is mediated by inducible NO synthase.

Lutgens et al. (1999) reported cardiac structural and functional changes after chronic myocardial infarction in the mouse.

Scherrer-Crosbie et al. (1999) described echocardiographic determination of risk area in a murine model of myocardial ischemia. Myocardial contrast echocardiography was performed before and after coronary artery ligation in anesthetized mice by intravenous injection of contrast microbubbles and transthoracic echo imaging. Time-video intensity curves were obtained for the anterior, lateral, and septal myocardial walls. After myocardial ischemia, myocardial contrast echocardiography defects were compared with the area of no perfusion measured by Evans blue staining.

Jones and Lefer (2001) described cardioprotective actions of acute HMG-CoA reductase inhibition in the setting of myocardial infarction.

Janssens et al. (2004) reported that cardiomyocyte-specific overexpression of NO synthase 3 (NOS3) improves left ventricular (LV) performance and reduces compensatory hypertrophy after myocardial infarction. The effect of cardiomyocyte-restricted overexpression of one NO synthase isoform, NOS3, on LV remodeling after myocardial infarction in mice was tested. LV structure and function before and after permanent left anterior descending (LAD) coronary artery ligation were compared in transgenic mice with cardiomyocyte-restricted NOS3 overexpression (NOS3-TG) and their wild-type littermates (WT). Before myocardial infarction, systemic hemodynamic measurements, echocardiographic assessment of LV fractional shortening (FS), heart weight, and myocyte width (as assessed histologically) did not differ in NOS3-TG and WT mice. The inotropic response to graded doses of isoproterenol was significantly reduced in NOS3-TG mice. One week after LAD ligation, the infarcted fraction of the LV did not differ in WT and NOS3-TG mice. Four weeks after myocardial infarction, however, end-systolic LV internal diameter (LVID) was greater, and FS and maximum and minimum rates of LV pressure development were less in WT than in NOS3-TG mice. LV weight/body weight ratio was greater in WT than in NOS3-TG mice.

LaPointe et al. (2004) found that inhibition of cyclooxygenase-2 (COX-2) improves cardiac function after myocardial infarction in the mouse. Myocardial infarction was produced by ligation of the LAD coro-

nary artery in mice. Two days later, mice were treated with a selective COX-2 inhibitor, or vehicle in drinking water for 2 weeks. After the treatment period, mice were subjected to two-dimensional M-mode echocardiography to determine cardiac function. Hearts were then analyzed for determination of infarct size, interstitial collagen content, brain natriuretic peptide (BNP) mRNA, myocyte cross-sectional area, and immunohistochemical staining for transforming growth factor (TGF) β and COX-2.

Shibuya et al. (2005) reported that *N*-acetyl-seryl-aspartyl-lysine-proline prevents renal insufficiency and matrix expansion in diabetic *db/db* mice.

Weinberg et al. (2005) found in coronary ligation experiments in mice, that rosuvastatin reduces experimental left ventricular infarct size after ischemia-reperfusion injury but not total coronary occlusion.

Yang et al. (2005) found that the infarct-sparing effect of A_{2A}-adenosine receptor activation is due primarily to its action on lymphocytes. Chimeric mice were created by bone marrow transplantation from A_{2A}AR-knockout or green fluorescent protein (GFP) donor mice to irradiated congenic C57BL/6 (B6) recipients. In the GFP chimeras, we were unable to detect GFP-producing cells in the vascular endothelium, indicating that bone-marrow-derived cells were not recruited to endothelium at appreciable levels after bone marrow transplantation and/or acute myocardial infarction. Injection of 5 or 10 μ g/kg of a potent and selective agonist of A_{2A} adenosine receptor had no effect on hemodynamic parameters but reduced infarct size in B6 mice after 45 min of LAD artery occlusion followed by 24 h of reperfusion.

Kanno et al. (2003) found **connexin43** to be a determinant of myocardial infarct size following coronary occlusion in mice.

Regulation of myocardial connexins during hypertrophic remodeling was reviewed by Teunissen et al. (2004).

Kuhlmann et al. (2006) reported that granulocyte colony stimulating factor (G-CSF), alone or in combination with stem cell factor (SCF), can improve hemodynamic cardiac function after myocardial infarction in mice and reduces inducible arrhythmias in the infarcted heart potentially via increased connexin43 expression and arteriogenesis.

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A.3.2.3

Occlusion of Coronary Artery in Anesthetized Dogs and Pigs

PURPOSE AND RATIONALE

The size of infarcts is studied after proximal occlusion of the left anterior descending coronary artery in open-chest dogs. Compounds potentially reducing infarct-size are tested. To delineate the post-mortem area at risk, coronary arteriograms are made after injection of a BaSO₄-gelatin mass into the left coronary ostium. The infarct's area is visualized with nitro-blue tetrazolium chloride in myocardial sections.

PROCEDURE

Dogs of either sex weighing approximately 30 kg are used. The animals are anesthetized by intravenous injection of pentobarbital sodium (bolus of 35 mg/kg followed by continuous infusion of 4 mg/kg/h). The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator. Arterial blood gases are checked, and the ventilation rate and/or oxygen flow rate are adjusted to achieve physiological blood gas values (P_{O₂}: 100–140 mm Hg, P_{CO₂}: 32–40 mm Hg, and pH 7.47). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously from lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral vein is connected to

a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular enddiastolic pressure (LVEDP) is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted.

Experimental Procedure

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left anterior descending coronary artery (LAD) is exposed. After reaching steady state conditions for the hemodynamic parameters (approx. 45 min), the LAD is ligated just below the first diagonal branch for 360 min. No attempt is made to suppress arrhythmic activity after the ligation.

The test substance or the vehicle (controls) is administered by intravenous bolus injection and/or continuous infusion. The schedule of administration may vary. Hemodynamic parameters are registered continuously during the whole experiment. At the end of the experiment, the animals are sacrificed with an overdose of pentobarbital sodium and the heart is dissected.

Preparation to Determine Area at Risk

Coronary arteriograms are made according to Schaper et al. (1979) to delineate the anatomic post-mortem area at risk. A purse-string suture is placed around the left coronary ostium in the sinus of Valsalva; a cannula is then placed in the ostium and the purse-string suture is tightened. Micronized BaSO₄ suspended in 12% gelatin solution (37°C) is injected under increasing pressure (2 min at 100 mm Hg, 2 min at 150 mm Hg and 2 min at 200 mm Hg). The heart is placed in crushed ice to gel the injectate. The right ventricle is removed and the left ventricle plus septum is cut into transverse sections (approx. 1 cm thick) from the apex to the level of the occlusion (near the base). From each slice angiograms are made with a X-ray tube at 40 kV to assess the post-mortem area at risk (by defect opacity: reduction of BaSO₄-filled vessels in infarct tissue).

Preparation to Determine Infarct Size

The slices are then incubated in p-nitro-blue tetrazolium solution (0.25 g/L in Sørensen phosphate buffer, pH 7.4, containing 100 mM D,L-maleate) in order to visualize the infarct tissue (blue/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of the infarct area.

Left ventricle and infarct area, and area at risk are measured by planimetry from projections of all slices with the exclusion of the apex and of the slice containing the ligature.

EVALUATION

Mortality and the different hemodynamic parameters are determined. Changes of parameters in drug-treated animals are compared to vehicle controls. The different characteristics are evaluated separately. Mean values \pm SEM of infarct area and of area at risk are calculated. Statistical analyses consist of regression and correlation analyses and of the Student's *t*-test. Results are considered significant at $p < 0.05$.

MODIFICATIONS OF THE METHOD

Nachlas and Shnitka (1963) described the macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity in dogs by staining the cardiac tissue with Nitro-BT [2,2'-di-*p*-nitrophenyl-5,5' diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride] yielding a dark blue formazan in viable muscle but not in necrotic muscle fibers.

Chiariello et al. (1976) compared the effects of nitroprusside and nitroglycerin on ischemic injury during acute myocardial infarction in dogs.

Black et al. (1995) studied the cardioprotective effects of heparin or *N*-acetylheparin in an *in vivo* dog model of myocardial and ischemic reperfusion injury. The hearts were removed after 90 min of coronary occlusion and a 6 h-reperfusion period. Area at risk was determined by the absence of Evans blue dye after perfusion of the aorta in a retrograde fashion and infarct zone by the absence of formazan pigment within the area at risk after perfusion of the circumflex coronary artery with triphenyltetrazolium chloride.

Reimer et al. (1985) tested the effect of drugs to protect ischemic myocardium in unconscious and conscious dogs. In the conscious model, dogs of either sex weighing 10–25 kg were anesthetized with thi-amyl sodium (30–40 mg/kg i.v.) and underwent thoracotomy through the 4th intercostal space. Heparin-filled polyvinyl chloride catheters were positioned in the aortic root, the left atrium via the left atrial appendage, and a systemic vein. A mechanical adjustable snare type occluder was placed around the proximal left circumflex coronary artery above or below the first marginal branch, so that temporary occlusion resulted in cyanosis of at least 75% of the inferior wall. The catheters and snare were either exteriorized or positioned in a subcutaneous pocket at the back of

the neck. Penicillin, 1000,000 units, and streptomycin, 1.0 g, were given i.m. for the first 4 postoperative days, and at least 7 days were allowed for recovery from surgery.

Dogs were fasted overnight prior to the study. After exteriorization and flushing of the catheters, 30–40 min were allowed for the animals to adjust to laboratory conditions. Morphine sulfate, 0.25 mg/kg, i.m., was given 30 min before occlusion, and an additional 0.25 mg/kg, i.v., was given 20 min later. Heart rate and aortic and left atrial pressures were monitored continuously. Permanent coronary occlusion was produced by a sudden one-stage tightening of the snare occluder. Drugs were administered by continuous i.v. infusion over 6 h. Hemodynamic measurements were taken 5 min before occlusion and 10, 25, 105, 180, and 360 min after occlusion.

Raberger et al. (1986) described a model of **transient myocardial dysfunction in conscious dogs**. Mongrel dogs, trained to run on a treadmill, were chronically instrumented with a miniature pressure transducer in the left ventricle and a hydraulic occluder placed around the circumflex branch of the left coronary artery. Two pairs of piezoelectrical crystals for sonomicrometry were implanted subendocardially to measure regional myocardial functions. Comparable episodes of regional dysfunction of the left coronary artery area during treadmill runs were found after partial left coronary artery stenosis induced by external filling of the occluder.

Hartman and Wartier (1990) described a model of **multivessel coronary artery disease** using conscious, chronically instrumented dogs. A hydraulic occluder was implanted around the left anterior descending coronary artery (LAD) and an Ameroid constrictor around the left circumflex coronary artery (LCCA). Pairs of piezoelectric crystals were implanted within the subendocardium of the LAD and LCCA perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion. Bolus injections of adenosine were administered daily via the left atrium to evaluate LAD and LCCA coronary reserve. After stenosis by the Ameroid constrictor, radioactive microspheres were administered to compare regional perfusion within normal myocardium to flow in myocardium supplied by the occluded or stenotic coronary arteries.

Holmborn et al. (1993) compared triphenyltetrazolium chloride staining versus detection of fi-

bronectin in experimental myocardial infarction in **pigs**.

Klein et al. (1995) used intact **pigs** and found myocardial protection by Na^+/H^+ exchange inhibition in ischemic reperfused hearts.

Klein et al. (1997) measured the time delay of cell death by Na^+/H^+ exchange inhibition in regionally ischemic, reperfused **porcine** hearts.

Garcia-Dorado et al. (1997) determined the effect of Na^+/H^+ exchange blockade in ischemic rigor contracture and reperfusion-induced hypercontracture in *pigs* submitted to 55 min of coronary occlusion and 5 h reperfusion. Myocardial segment length analysis with ultrasonic microcrystals was used to detect ischemic rigor (reduction in passive segment length change) and hypercontracture (reduction in end-diastolic length).

Symons et al. (1998) tested the attenuation of regional dysfunction in response to 25 cycles of ischemia (2 min) and reperfusion (8 min) of the left circumflex coronary artery in **conscious swine** after administration of a Na^+/H^+ exchange inhibitor. The animals were instrumented to measure arterial blood pressure, regional myocardial blood flow (colored microspheres), systolic wall thickening in the normally perfused left anterior descending and left circumflex coronary artery regions (sonomicrometry), left circumflex coronary artery blood flow velocity (Doppler) and reversibility to occlude the left circumflex coronary artery (hydraulic occluder).

Etoh et al. (2001) studied myocardial and interstitial matrix metalloproteinase activity after acute myocardial infarction in **pigs**.

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A.3.2.4

Acute Ischemia by Injection of Microspheres in Dogs

PURPOSE AND RATIONALE

Severe left ventricular failure is induced by repeated injections of 50 μm plastic microspheres into the left main coronary artery of anesthetized dogs. Hemodynamic measurements are performed under these conditions testing drugs which potentially improve cardiac performance. The test can be used to evaluate the influence of drugs on myocardial performance during acute ischemic left ventricular failure in dogs.

PROCEDURE

Dogs of either sex weighing approximately 30 kg are anesthetized by an intravenous bolus injection of 35–40 mg/kg pentobarbital sodium continued by an infusion of 4 mg/kg/h. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator and controlled by measuring end-expiratory CO₂ concentration as well as blood gases. Two peripheral veins are cannulated for the administration of narcotic (brachial vein) and test compounds (saphenous vein). The ECG is recorded continuously in lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of the right femoral vein is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (Gould PC 350) is inserted via the left carotid artery. Left ventricular enddiastolic pressure (LVEDP) is measured on a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate (HR) is counted. To measure right ventricular pressure, a Millar microtip catheter is inserted via the right femoral vein. Systolic, diastolic and mean pulmonary artery pressure (PAP), mean pulmonary capillary pressure, and cardiac output are measured by a thermodilution technique using a Cardiac Index Computer (Gould SP 1435) and a balloon-tipped triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left circumflex coronary artery (LCX) is exposed. To measure coronary blood flow, an electromagnetic flow probe (Hellige Recomed) is placed on the proximal part of the LCX.

Polystyrol microspheres (3M Company, St. Paul, Minnesota, USA) with a diameter of $52.5 \pm 2.24 \mu\text{m}$ are diluted with dextran 70, 60 mg/ml and saline at a concentration of 1 mg microspheres/ml (1 mg = approx. 12,000 beads). For administration of microspheres, an angiogram catheter (Judkins-Schmidt Femoral-Torque, William Cook, Europe Aps. BP 7) is inserted into the left ostium via the left femoral artery.

Induction of Failure

The microspheres are injected through the angiogram catheter into the left ostium initially as 10 ml and later as 5 ml boluses about 5 min apart. The microsphere in-

jections produce stepwise elevations of LVEDP. Embolization is terminated when LVEDP has increased to 16–18 mm Hg and/or PAPm has increased to 20 mm Hg and/or heart rate has reached 200 beats/min. The embolization is completed in about 70 min and by injection of an average dose of 3–5 mg/kg microspheres. Hemodynamic variables are allowed to stabilize after coronary embolization for at least 30 min.

Experimental Course

The test substance or the vehicle (controls) is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application.

Recordings are obtained

- before embolization
- after embolization
- before administration of test compound
- 5, 30, 45, 60, 90, 120 and, eventually, 150 and 180 min following administration of test drug. At the end of the experiment, the animal is sacrificed by an overdose of pentobarbital sodium.

EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formula:

stroke volume [ml/s],

$$SV = \frac{\text{cardiac output}}{\text{heart rate}}$$

tension index [mmHg/s],

$$IT = \frac{BP_s \times \text{heart rate}}{1000}$$

Coronary vascular resistance [mm Hgmin/ml],

$$CVR = \frac{BP_m \times RAP_m}{CBF}$$

total peripheral resistance [dyns/cm⁵],

$$TPR = \frac{BP_m \times RAP_m}{\text{cardiac output}} \times 79.9$$

Pulmonary artery resistance [dyn s/cm⁵],

$$PAR = \frac{PAP_m - PCP_m}{\text{cardiac output}} \times 79.9$$

right ventricle work [kgm/min],

$$RVW = (PAP_m - RAP_m) \times \text{cardiac output} \times 0.0136$$

left ventricle work [kgm/min],

$$LVW = (BPm - LVEDP) \times \text{cardiac output} \times 0.0136$$

left ventricular myocardial oxygen consumption [ml O₂/min/100 g],

$$\begin{aligned} MVO_2 = & K_1 (BPs \times HR) \\ & + K_2 \frac{(0.8BPs + 0.2BPd) \times HR \times SV}{BW} \\ & + 1.43 \end{aligned}$$

$$K_1 = 4.08 \times 10^{-4}$$

$$K_2 = 3.25 \times 10^{-4}$$

BPs = systolic blood pressure [mm Hg]

BPd = diastolic blood pressure [mm Hg]

BPm = mean blood pressure [mm Hg]

CBF = coronary blood flow in left circumflex coronary artery [ml/min]

RAPm = mean right atrial pressure [mm Hg]

PAPm = mean blood pressure A. pulmonalis [mm Hg]

PCPm = mean pulmonary capillary pressure

HR = heart rate [beats/min]

SV = stroke volume [ml]

BW = body weight [kg]

Changes of parameters in drug-treated animals are compared to vehicle controls; statistical significance of the differences is calculated with the Student's *t*-test.

Mean embolization times, doses of microspheres and number of microsphere applications are evaluated.

MODIFICATIONS OF THE METHOD

Gorodetskaya et al. (1990) described a simple method to produce acute heart failure by coronary vessel embolization with microspheres in rats.

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A.3.2.5

Influence on Myocardial Preconditioning

PURPOSE AND RATIONALE

Damage to the mammalian heart produced by prolonged ischemia and reperfusion can be reduced by “preconditioning” the myocardium via a brief cycle of ischemia and reperfusion prior to the protracted ischemic event. Ischemic preconditioning has been shown to decrease infarct size and increase recovery of post-ischemic ventricular function (Murray et al. 1986), and to reduce leakage of cellular marker proteins indicative for cardiac myocyte death (Volovsek et al. 1992). In addition, preliminary preconditioning also attenuates cardiac arrhythmia associated with subsequent occlusion and reperfusion (Vegh et al. 1990).

The mechanistic basis of this phenomenon is under discussion (Parratt 1994; Parratt and Vegh 1994). Adenosine receptor involvement in myocardial protection after ischemic preconditioning in rabbits has been shown by Baxter et al. (1994). Adenosine (A₁ receptor) antagonists have been demonstrated to block the protection produced by preconditioning (Liu et al. 1991), and short term administration of adenosine was shown to simulate the protective effects of ischemic preconditioning (Toombs et al. 1993). These observations together suggest that adenosine is generated by the short preconditioning ischemia. Other recent pharmacological studies (Gross and Auchampach 1992; Yao and Gross 1994) indicate the involvement of the ATP-sensitive potassium channel. Recent investigations indicate that an increase of NO production after ACE inhibitors may be a part of the protective mechanism (Linz et al. 1992, 1994). Moreover, the involvement of prostanoids and bradykinin in the preconditioning process has been discussed (Wiemer et al. 1991). Gho et al. (1994) found a limitation of myocardial infarct size in the rat by transient renal ischemia, supporting the hypothesis that the mechanism leading to cardiac protection by ischemic preconditioning may not only reside in the heart itself.

PROCEDURE

New Zealand rabbits of either sex weighing 2.5–3.5 kg are initially anesthetized with an intramuscular injection of ketamine (50 mg/ml) /xylazine (10 mg/ml) solution at a dose of 0.6 ml per kg body weight. A tracheotomy is performed to facilitate artificial respiration. The left external jugular vein is cannulated to permit a constant infusion (0.15–0.25 ml/min) of xylazine (2 mg/ml in heparinized saline) to assist in

maintaining anesthesia and fluid volume. Anesthesia is also maintained by i.m. injections (0.4–0.6 ml) of ketamine (80 mg/ml) and xylazine (5 mg/ml) solution. After the xylazine infusion is started, animals are respired with room air at a tidal volume of 10 ml/kg and a frequency of 30 inflations per min (Harvard Apparatus, USA). Thereafter, ventilation is adjusted or inspiratory room air is supplemented (5% CO₂/95% O₂) to maintain arterial blood chemistry within the following ranges: pH 7.35–7.45, P_{CO2} 25–45 mm Hg, P_{O2} 90–135 mm Hg. The right femoral artery and vein are isolated and catheterized for measurement of arterial pressure and administration of drugs, respectively.

A thoracotomy is performed in the fourth intercostal space, and the lungs are retracted to expose the heart. The pericardium is cut to expose the left ventricle, and a solid-state pressure transducer catheter (e. g., MicroTip 3F, Millar Instruments, Houston, USA) is inserted through an apical incision and secured to enable measurement of pulsatile left ventricular pressure. The maximal rate of increase in left ventricular pressure (LVdP/dt max) is determined by electronic differentiation of the left ventricular pressure wave form. A segment of 4–0 prolene suture is looped loosely around a marginal branch of the left main coronary artery to facilitate coronary occlusion during the experiment. Needle electrodes are inserted subcutaneously in a lead II configuration to enable recording of an ECG in order to determine heart rate and help confirm the occurrence of ischemia (ST segment elevation) and reperfusion of the myocardium distal to the coronary occlusion. Continuous recording of pulsatile pressure, ECG, heart rate, and LVdP/dt are simultaneously displayed on a polygraph (e. g. Gould chart recorder, Gould Inc., Valley View, USA) and digitized in real time by a personal computer. Hemodynamic data are condensed for summary and later statistical analysis.

Ischemic preconditioning is induced by tightening the prolene loop around the coronary artery for 5 min and then loosening to reperfuse the affected myocardium for 10 min prior to a subsequent 30 min occlusion. After surgical preparation, and prior to 30 min of occlusion, rabbits are randomly selected to receive ischemic preconditioning, no preconditioning, or ischemic preconditioning plus treatment with test drugs. After 30 min of occlusion, the ligature is released and followed by 120 min of reperfusion. Occlusion is verified by epicardial cyanosis distal to the suture, which is usually accompanied by alterations in hemodynamics and ECG. Reperfusion is validated by return of original color. Systemic hemodynamics are summarized

for each experimental period. The experiment is terminated after 120 min of reperfusion, and the heart is excised for determinations of infarct size and area at risk.

Immediately before the animal is sacrificed, the marginal branch of the left coronary artery is reoccluded and India ink is rapidly injected by syringe with a 18-g needle into the left ventricular chamber to demarcate blackened normal myocardium from unstained area at risk. After the rabbits are sacrificed, the heart is removed and sectioned in a breadloaf fashion from apex to base perpendicular to the long axis. The right ventricle is removed from each slice leaving only the left ventricle and septum. After each slice is weighed, the portions are washed and incubated in a phosphate buffered saline solution of triphenyl tetrazolium chloride (1 g/ml, Sigma) for 10–15 min. Salvaged myocardium in the area at risk stains brick red, whereas infarcted tissue remains unaltered in color. Slices are then placed between sheets of Plexiglas and the areas (normal, risk, infarct) of each slice are traced on a sheet of clear acetate. Traces are then digitized and analyzed using computerized planimetry to compare the relative composition of each slice with respect to normal tissue, area at risk, and infarcted myocardium. Planimetry is performed with a computerized analysis system, e. g., Quantimet 570C image analysis system (Leica, Deerfield, USA).

Surface areas of normal tissue, area at risk, and infarcted myocardium on both sides of each slide are averaged for the individual slide. The contribution of each slide to total infarcted and area at risk (%) and area at risk as a percentage of total left ventricular mass for the entire left ventricle is prorated by the weight of each slice (Garcia-Dorado et al. 1987). By adding the adjusted contributions from each slice to infarcted tissue, area at risk, and left ventricular mass, a three-dimensional mathematical representation of total myocardial infarct size and risk zone can be calculated for each rabbit, and a mean tabulated for each treatment group for statistical comparison.

EVALUATION

All data are presented as mean \pm SD. Systemic hemodynamic data are analyzed by ANOVA using Statistica/W software. Means are considered significantly different at $p < 0.05$.

MODIFICATIONS OF THE METHOD

Li et al. (1990) found in dog experiments that preconditioning with one brief ischemic interval is as effective as preconditioning with multiple ischemic periods.

In contrast, Vegh et al. (1990) found in other dog experiments that two brief preconditioning periods of coronary occlusion, with an adequate period of reperfusion between, reduce the severity of arrhythmias.

Yang et al. (1996) found a second window of protection after ischemic preconditioning in conscious rabbits which minimizes both infarction and arrhythmias.

Late preconditioning against myocardial stunning in conscious pigs together with an increase of heat stress protein (HSP)70 was described Sun et al. (1995).

Szilvassy et al. (1994) described the anti-ischemic effect induced by ventricular overdrive pacing as a conscious rabbit model of preconditioning. Rabbits were equipped with right ventricular electrode catheters for pacing and intracavitary recording and polyethylene cannulae in the left ventricle and right carotid artery to measure intraventricular pressure and blood pressure. One week after surgery in conscious animals, ventricular overdrive pacing at 500 beats/min over 2, 5, or 10 min resulted in an intracavitary S-T segment elevation, shortening of ventricular effective refractory period, decrease in maximum rate of pressure development and blood pressure, and increase in left ventricular end-diastolic pressure proportional to the duration of stimulus. A 5-min preconditioning ventricular overdrive pacing applied 5 or 30 min before a 10-min ventricular overdrive pacing markedly attenuated ischemic changes, whereas a 2-min ventricular overdrive pacing had no effect.

The ventricular overdrive pacing induced preconditioning effect was lost in atherosclerotic rabbits (Szilvassy et al. 1995), however, delayed cardiac protection could be induced in these animals (Szekeres et al. 1997).

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A.3.2.6

MRI Studies of Cardiac Function

PURPOSE AND RATIONALE

Magnetic resonance imaging (MRI) is the preferred technique for the visualization of lesions in the brain and spinal cord of patients with MS. It visualizes the resonance signals of tissue protons when they are placed in a time-varying strong magnetic field. The most frequently used parameters measured in MS are the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1-mm-thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T_2 images are sensitive to water and, because all pathological alterations in MS brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualization of the spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T_1 values, whereas relatively loose structures, such as grey matter or lesions, have higher T_1 values.

To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected (0.1 – 0.3 mmol kg^{-1}) and, in areas of increased blood–brain barrier permeability, leaks into the brain parenchyma, causing local enhancement of the T_1 -weighted signal intensity.

A third important MRI technique in MS is magnetization transfer ratio (MTR) imaging. The MTR of a given tissue is defined as the ratio of free protons versus protons bound to tissue macromolecules.

MRI has emerged as a highly accurate and quantitative tool for the evaluation of cardiac function (Peshock et al. 1996).

Al-Shafei et al. (2000a, 2002b) performed MRI analysis of cardiac cycle events in diabetic rats and tested the effect of angiotensin-converting enzyme inhibition.

PROCEDURE

Diabetes was induced in Wistar rats at an age of 7, 10, and 13 weeks. The rats were anesthetized using 1%–2% halothane in oxygen, their blood glucose levels were checked. They were then given a single intraperitoneal injection of streptozotocin 65 mg/kg body weight. The control rats received sham injections of the citrated buffer when they were 7 weeks old. One diabetic group was treated with 2 g/l captopril in the drinking water

For **MR imaging**, rats were anesthetized using 1%–2% halothane in oxygen, weighed and their systolic blood pressures measured non-invasively using a rat tail blood pressure monitor both before and after imaging sessions to confirm physiological stability. Electrocardiographic (ECG) monitoring used shielded subcutaneous electrodes and a Tektronix 2225 oscilloscope. The cine imaging protocols were performed with the anesthetized animal placed in a specially designed home-built half-sine-spaced birdcage radiofrequency (RF) probe unit contained within a cylindrical plastic holder fitted within a gradient set of internal diameter 11 cm. The RF probe unit was made up of a half-sine spaced birdcage RF probe of internal diameter of 4.5 cm with open ends, an RF shield consisting of a cylinder of copper gauze surrounding and sliding over the birdcage, a tuning capacitor and a coaxial cable to carry the RF (Ballon et al. 1990). The assembly included ECG leads, attachment plugs for the ECG leads and a unit to anchor anesthetic delivery tubes near the nose of the animal. All experiments used a 2 T Oxford Instruments (UK) superconducting magnet with a horizontal internal bore of 31 cm. A gated cine protocol synchronized line acquisition to set times following alternate electrocardiographic R waves. This acquisition was then repeated at the same slice position at 12 equally incremented times through the cardiac cycle. This sequence in turn was repeated for each of the 128 lines to generate each 128×128 image, which itself was acquired twice for signal averaging. The preceding procedure was in turn repeated 12 times to obtain signal-averaged images for every one of the 12 contiguous transverse slices examined. Each imaging session therefore required $(128 \times 12 \times 2 \times 2)$ times the

cardiac cycle duration. The effective repeat time (TR) was approximately 13 ms. The short echo time (TE) of 4.3 ms reduced motion artifacts and ensured good contrast between blood and myocardium.

EVALUATION

The image data were transferred from the MRI console using in-house hardware and software to remote UNIX workstations for quantitative analysis using in-house software based on CaMReS libraries (CaMReS, Dr N. J. Herrod, Herchel Smith Laboratory for Medicinal Chemistry, University of Cambridge).

MODIFICATIONS OF THE METHOD

Itter et al. (2004) used non-invasive MRI techniques in a model of chronic heart failure in spontaneously hypertensive rats.

Bryant et al. (1998) and Franco et al. (1999) described MRI and invasive evaluation of development of heart failure in transgenic mice with myocardial expression of tumor necrosis factor- α .

Wiesmann et al. (2002) reported analysis of right ventricular function in healthy mice and a murine model of heart failure by *in vivo* MRI.

Kraitichman et al. (2003) described quantitative ischemia detection during cardiac magnetic resonance stress testing by use of fast harmonic phase MTI (FastHARP) in dogs.

Reddy et al. (2004) discussed the feasibility of a porcine model of healed myocardial infarction by integration of cardiac MRI with three-dimensional electroanatomic mapping to guide left ventricular catheter manipulation.

Pelzer et al. (2005) reported that the estrogen receptor- α agonist 16 α -LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. Improved left ventricular function upon 16 α -LE2 treatment was also observed in cardiac MRI studies.

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A.3.2.7

MRI Studies After Heart and Lung Transplantation

PURPOSE AND RATIONALE

Acute cardiac allograft rejection continues to be the cause of graft loss and contributes to the morbidity and mortality after cardiac transplantation. Endomyocardial biopsy is used routinely for cardiac transplant rejection surveillance. A sensitive and non-invasive method for detecting rejection is desirable. Kanno et al. (2001) developed a rat model of heterotopic heart and lung transplantation for MRI experiments. Allograft transplantations were performed with syngeneic transplantations serving as controls. MR images were obtained with a gradient echo method.

PROCEDURE

Animals

All rats used in the experiments were male, 2–3 months of age, and weighed 220–250 g each. Ani-

mals were housed individually and provided with food and water ad libitum. Inbred Brown Norway (BN; RT1^b) and DA (RT1^a) rats were obtained from Harlan Sprague Dawley (Indianapolis, Ind).

Heart and Lung Transplantation

Under anesthesia with injection of 35 mg/kg body weight of sodium pentobarbital IP, 500 U/kg body weight of heparin was injected. In the syngeneic group, an en bloc donor heart and lung were taken from a BN rat and transplanted to another BN rat. In the allogeneic group, a graft from a DA rat was transplanted to a BN rat. This group was divided into two groups: one group was treated with 3 mg/kg per day ciclosporin (CsA), and the other group was not given CsA. Graft survival was monitored every day by palpating contraction of the transplanted heart.

Operative procedures have been described by Kanno et al. (2000). In brief, after the chest wall of the donor rat was opened, the left lung was ligated and excised. The azygos vein with the left superior and right superior venae cavae was ligated and divided. The descending thoracic aorta was transected, and 10 ml of cold University of Wisconsin solution (UW solution, Dupont Pharma) was infused into the inferior vena cava until the fluid draining from the aorta was clear, followed by ligation and division of the inferior vena cava. The ascending aorta was dissected and transected at the portion between the left common carotid artery and the left subclavian artery, followed by ligation and division of the right brachiocephalic artery and the left common carotid artery. After removal of the heart and lung from the donor, the right lung was washed 3 times through the bronchus with UW solution containing penicillin G. The grafts were then placed into cold UW solution for ≈ 5 min until transplantation. Next, the left inguinal portion of the recipient rat was opened and dissected to make enough space for the transplanted organs. The left lower part of the abdominal wall was opened in a transverse fashion from the left femoral vessels to the midline. The abdominal organs were retracted to the right, and both the aorta and the inferior vena cava just beyond the bifurcation were dissected. The vessels were clamped, and an appropriate opening of the aorta was made to receive the aorta of the graft in an end-to-side fashion. Rhythmic heartbeats commenced spontaneously as the heart and the lung regained circulation after removal of the clamp. After hemostasis of the surgical field, the abdominal wall was sutured, with care taken not to kink or obstruct the aorta of the graft.

MRI EXPERIMENTS

MRI measurements were carried out on a 4.7-T/40-cm Bruker AVANCE DRX MR instrument equipped with 15-cm, 10-gauss/cm shielded gradients. *In vivo* MR images of transplanted heart-lung were obtained over a period of 24 h after infusion of dextran-coated ultrasmall superparamagnetic iron oxide (USPIO) particles. The imaging sequence consisted of a gradient echo sequence, triggered to ECG and ventilator (60 strokes/min, 10 ml/kg), with TR/TE 500/10 ms, flip angle equal to Ernst angle, slice thickness 1 mm, field of view 6.0 cm, data matrix size 256×130 (zero-filled to 256×256), and scan time 5 min. ECG leads were placed on both of the hind limbs of the rat with the transplant to pick up the heartbeat from the transplanted heart more effectively. The change of MRI signal intensity was measured in whole ventricular wall in each transplanted heart. The MR signal intensity of the heart was normalized to that of the leg muscle, because USPIO particles are not readily taken up by muscular tissue, according to Gellissen et al. (1999)

Dextran-coated USPIO particles were synthesized according to the method of Palmacci and Josephson (1993) with slight modifications (Dodd et al. 1999). The MR relaxivities R_1 (spin-lattice relaxation rate constant, $1/T_2$, per mol of Fe in USPIO) and R_2 (spin-spin relaxation rate constant, $1/T_2$, per mol of Fe in USPIO) measured at 4.7 T were 3.8×10^4 and 9.1×10^4 (mol/l)/s, respectively. For *in vivo* studies, dextran-coated USPIO particles were dialyzed against PBS solution and diluted to a concentration of 18 μ mol Fe/ml, and 0.8 ml of the suspension (i. e., ≈ 3 mg Fe/kg body weight) was injected intravenously for each study.

At 6 days after transplantation, dextran-coated USPIO particles were injected intravenously as mentioned above, and the animals were subjected to MRI. Then 24 h later, these animals were again placed inside the magnet and scanned. The regions of interest were defined manually with Bruker software. MR signal intensity in the entire ventricular wall in the plane was measured. After injection of USPIO particles at post-operative day (POD) 6, animals with allotransplants were given CsA for 4 (POD 7 to 10) or 7 (POD 7 to 13) days and reinjected with USPIO particles on POD 14.

Pathological Analysis and Immunohistochemistry

After an MR experiment was completed, the transplanted hearts were extirpated, fixed in 3.7% formaldehyde, and embedded in paraffin for 5- μ m sections. Hematoxylin-eosin staining and Perl's Prussian blue staining were performed in the Transplantation Pathol-

ogy Laboratory of the University of Pittsburgh Medical Center. Histological analysis for pathological grading of heart rejection, which is based on the criteria established by the International Society for Heart and Lung Transplantation, was also performed by this laboratory in a blinded manner. Monoclonal anti-rat macrophage antibody (ED1, Serotec) was used as a primary antibody for macrophages. Immunohistochemistry was carried out with the ABC staining system (Santa Cruz Biotechnology) according to the manufacturer's protocol.

EVALUATION

The results are presented as mean \pm SD. The results were analyzed by ANOVA with StatView software (SAS Institute). A value of $P < 0.05$ was considered to be statistically significant.

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A.3.3

Ex Vivo Methods

A.3.3.1

Plastic Casts from Coronary Vasculature Bed

PURPOSE AND RATIONALE

Prolonged administration of coronary drugs has been shown to increase the number and size of interarterial collaterals of dogs and pigs after coronary occlusion (Vineberg et al. 1962; Meesmann and Bachmann 1966). An increased rate of development of collateral arteries was observed after physical exercise in dogs (Schaper et al. 1965), as well as after chronic administration of coronary dilating drugs (Lumb and Hardy 1963). An even more effective stimulus for collateral development is an acute or gradual occlusion of one

or several major coronary branches. Filling the arterial coronary bed with a plastic provides the possibility to make the collaterals visible and to quantify them (Schmidt and Schmier 1966; Kadatz 1969).

PROCEDURE

Dogs weighing 10–15 kg are anesthetized with pentobarbital sodium 30 mg/kg i.v. They are respiration artificially and the thorax is opened. After opening of the pericard, Ameroid cuffs are placed around major coronary branches. Gradual swelling of the plastic material occludes the lumen within 3–4 weeks. The dogs are treated daily with the test drug or placebo. After 1 week recovery period they are submitted to exercise on a treadmill ergometer. After 6 weeks treatment, the animals are sacrificed, the heart removed and the coronary bed flushed with saline. The liquid plastic Araldite is used to fill the whole coronary tree from the bulbous aortae. The aortic valves are glued together in order to prevent filling of the left ventricle. Red colored Araldite is used to fill the arterial tree. The venous part of the coronary vasculature can be filled with blue colored Araldite from the venous sinus. The uniformity of the filling pressure, the filling time, and the viscosity of the material are important. Polymerization is complete after several hours. Then, the tissue is digested with 35% potassium hydroxide. The method gives stable preparations which can be preserved for a long time.

EVALUATION

Plastic casts from drug treated animals are compared with casts from dogs submitted to the same procedure without drug treatment.

CRITICAL ASSESSMENT OF THE METHOD

The procedure allows impressive demonstration of the formation of arterial collaterals. The results of post mortem Araldite impregnation agree with the functional results of experimental coronary occlusion.

MODIFICATIONS OF THE METHOD

Boor and Reynolds (1977) described a simple planimetric method for determination of left ventricular mass and necrotic myocardial mass in postmortem hearts.

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A.4 Calcium Uptake Inhibiting Activity

A.4.0.1

General Considerations

Cellular calcium flux is regulated by receptor-operated and voltage-dependent channels, which are sensitive to inhibition by calcium entry blockers. The term calcium antagonist was introduced by Fleckenstein (1964, 1967) when two drugs, prenylamine and verapamil, originally found as coronary dilators in the LANGENDORFF-experiment, were shown to mimic the cardiac effects of simple Ca^{2+} -withdrawal, diminishing Ca^{2+} -dependent high energy phosphate utilization, contractile force, and oxygen requirement of the beating heart without impairing the Na^+ -dependent action potential parameters. These effects were clearly distinguishable from β -receptor blockade and could promptly be neutralized by elevated Ca^{2+} , β -adrenergic catecholamines, or cardiac glycosides, measures that restore the Ca^{2+} supply to the contractile system. In the following years many Ca^{2+} -antagonists were introduced to therapy. Specific Ca^{2+} -antagonists interfere with the uptake of Ca^{2+} into the myocardium and prevent myocardial necrotization arising from deleterious intracellular Ca^{2+} overload. They act basically as spe-

cific inhibitors of the slow transsarcolemmal Ca^{2+} influx but do not or only slightly affect the fast Na^+ current that initiates normal myocardial excitation.

Calcium channels and the sites of action of drugs modifying channel function have been classified (Bean 1989; Porzig 1990; Tsien and Tsien 1990; Spedding and Paoletti 1992).

Nomenclature and structure–function relationships of voltage-gated calcium channels were reviewed by Catterall et al. (2005).

Kochegarow (2003) reviewed the therapeutic application of modulators of voltage-gated calcium channels.

Four main types of voltage dependent calcium channels are described:

1. L type (for long lasting),
2. T type (for transient),
3. N type (for neuronal), and
4. P type (for Purkinje cells).

They differ not only by their function (Dolphin 1991) and localization in tissues and cells but also by their sensitivity to pharmacological agents (Ferrante and Triggle 1990; Dascal 1990; Kitamura et al. 1997) and by their specificity to radioligands.

The widely distributed **L-type channels** exist in isoforms (L1, 2, 3, 4) and consist of several subunits, known as α_1 , α_2 , β , γ , δ . They are sensitive to dihydropyridines, phenylalkylamines or benzothiazepines, but insensitive to ω -conotoxin and ω -agatoxin. The segments required for antagonist binding have been analyzed (Peterson et al. 1996, Schuster et al. 1996; Mitterdorfer et al. 1996; Hockerman et al. 1997; Striessnig et al. 1998; Catterall 1998).

Berjukow et al. (2000) discussed the molecular mechanism of calcium channel block by isradipine.

Striessnig et al. (2004) described the role of L-type Ca^{2+} channels in Ca^{2+} channelopathies.

The **T-type channels** are located mainly in the cardiac sinoatrial node and have different electrophysiological characteristics from L-type channels (Massie 1997; Perez-Reyes et al. 1998).

Reviews of molecular physiology of low-voltage-activated T-type calcium channels were given by Perez-Reyes (2003, 2006).

N- and P-type calcium channels blockers occur in neuronal cells and are involved in neurotransmitter release (Olivera et al. 1987; Bertolino and Llinás 1992; Mintz et al. 1992; Woppmann et al. 1994; Diversé-Pierluissi et al. 1995; Miljanich and Ramachandran 1995; Fisher and Bourque 1996; Ikeda 1996; Ertel et al. 1997; Sinnegger et al. 1997).

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A.4.1

In Vitro Methods

A.4.1.1

^3H -Nitrendipine Binding in Vitro

PURPOSE AND RATIONALE

Radiolabeled dihydropyridine calcium channel antagonists such as ^3H -nitrendipine (Salter and Grover 1987; Campiani et al. 1996) are selective ligands for a drug receptor site associated with the voltage-dependent calcium channel. A constant concentration of the radioligand ^3H -nitrendipine (0.3–0.4 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine cerebral cortices. If the test drug exhibits any affinity to calcium channels, it is able to compete with the radioligand for channel binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug.

PROCEDURE

Materials and solutions:

| | | |
|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------|
| Preparation buffer | Tris-HCl pH 7.4 | 50 mM |
| Incubation buffer | Tris-HCl Genapol pH 7.4 | 50 mM 0.001% |
| Radioligand | ^3H -nitrendipine Specific activity 2.59–3.22 TBq/mmol (70–87 Ci/mmol) (NEN) | |
| For inhibition of ^3H -nitrendipine binding in non-specific binding Experiments | Nifedipine (Sigma) | |

Two freshly slaughtered bovine brains are obtained from the slaughterhouse and placed in ice-cold preparation buffer. In the laboratory, approx. 5 g wet weight of the two frontal cerebral cortices are separated from the brains.

Membrane Preparation

The tissue is homogenized (glass Teflon potter) in ice-cold preparation buffer, corresponding to 1 g cerebral wet weight/50 ml buffer, and centrifuged at 48,000 g (4°C) for 10 min. The resulting pellets are resuspended in approx. 270 ml preparation buffer, and the homogenate is centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebral cortex wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at –77°C. Protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 48,000 g (4°C) for 10 min. The resulting pellets are resuspended in a volume of ice-cold incubation buffer, yielding a membrane suspension with a protein content of 0.6–0.8 mg/ml. After homogenization (glass Teflon potter), the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

As 1,4-dihydropyridines tend to bind to plastic material, all dilution steps are done in glass tubes.

For each concentration samples are prepared in triplicate. The total volume of each incubation sample is 200 μl (microtiter plates).

Saturation Experiments

total binding:

- 50 μl ^3H -nitrendipine (12 concentrations, 5×10^{-11} – 4×10^{-9} M)
- 50 μl incubation buffer non-specific binding:
- 50 μl ^3H -nitrendipine (4 concentrations, 5×10^{-11} – 4×10^{-9} M)
- 50 μl nifedipine (5×10^{-7} M)

Competition Experiments

- 50 μl ^3H -nitrendipine (1 constant concentration, 3 – 4×10^{-10} M)
- 50 μl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10} – 10^{-3} M)

The binding reaction is started by adding 100 μl membrane suspension per incubation sample (0.6–0.8 mg protein/ml). The samples are incubated for 60 min in a bath shaker at 25°C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fibre filters. Thereby the membrane-bound is separated from the free radioactivity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding - non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^3H -nitrendipine versus non-labeled drug by a computer-supported analysis of the binding data.

$$\frac{K_D \text{ } ^3\text{H} \times \text{IC}_{50}}{K_D \text{ } ^3\text{H} + [^3\text{H}]}$$

IC_{50} = concentration of the test drug, which displaces 50% of specifically bound ^3H -nitrendipine in the competition experiment

$[^3\text{H}]$ = concentration of ^3H -nitrendipine in the competition experiment.

$K_D \text{ } ^3\text{H}$ = dissociation constant of ^3H -nitrendipine, determined from the saturation experiment.

The K_i -value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data:

- nifedipine $K_i = 2$ – 4×10^{-9} mol/l

MODIFICATIONS OF THE METHOD

$[^3\text{H}](+)\text{-PN200-100}$ (israpidine) has been used by many authors as the labeled ligand for binding experiments (Grassegger et al. 1989; Nokin et al. 1990; Striessnig et al. 1991; Yaney et al. 1991; Miwa et al. 1992; Ichida et al. 1993; Kalasz et al. 1993; Ikeda et al. 1994; Rutledge and Triggle 1995; Shimasue et al. 1996; He et al. 1997; Natale et al. 1999; Matthes et al. 2000; Peri et al. 2000).

Yaney et al. (1991) performed binding experiments with $[^3\text{H}](+)\text{-PN200-110}$ to membranes of RINm5F cells.

The RINm5F pancreatic β -cells were grown on plastic culture flasks in medium RPMI 1640 supplemented with 10% fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ of streptomycin and 100 U/ml penicillin. The cells were kept in humidified incubators at 37°C in 5% CO_2 -95% air. Maintenance flasks were subcultured every 4–5 days at $\sim 80\%$ confluency. For binding experiments of $[^3\text{H}](+)\text{-PN200-110}$ to membranes, cells were removed from the flasks by incubation for 15–20 min in 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] and 1 mM EGTA (pH 7.4) at 4°C. The osmotically ruptured cells were homogenized with a Brinkmann Polytron followed by centrifugation at 20,000 g for 15 min. The pellet was resuspended and washed twice. The final pellet was resuspended in 1 mM CaCl_2 and 20 mM MOPS (pH 7.4) at a protein concentration of 0.2–0.4 mg/ml. Membranes were incubated at room temperature with (+)- $[^3\text{H}]\text{PN200-110}$ in a final volume of 500 μl (Weiland and Oswald 1985). Bound and free radioligand were separated by vacuum filtration through a glass fiber filter (no. 32, Schleicher and Schüll) followed by three washes of the filter with 3 ml incubation buffer. Non-specific binding was determined in the presence of 1 μM nitrendipine. Radioactivity retained by the filters was determined by liquid scintillation counting. The concentration of free radioligand was calculated by scintillation counting of aliquots of incubation mixtures under nonspecific conditions before and after centrifugation.

Several other calcium entry blockers, such as nimodipine, diltiazem, verapamil and desmethoxyverapamil, have been labeled and used for binding studies in order to elucidate the calcium channel recognition sites and may be used for further classification of calcium antagonists (Ferry and Glossmann 1982; Glossmann et al. 1983; Goll et al. 1984; Lee et al. 1984; Glossmann et al. 1985; Schoemaker and Langer 1985; Ruth et al. 1985; Reynolds et al. 1986).

Vaghy et al. (1987) identified of a 1,4-dihydropyridine- and phenylalkylamine-binding polypeptide in calcium channel preparations.

Naito et al. (1989) described photoaffinity labeling of the purified skeletal muscle calcium antagonist receptor by [³H]azidobutyryl diltiazem.

Watanabe et al. (1993) reported that azidobutyryl clentiazem labels the benzothiazepine binding sites in the α_1 subunit of the skeletal muscle calcium channel. Tissue heterogeneity of calcium channel antagonist binding sites has been demonstrated by Gould et al. (1983).

Photoaffinity labeling of the cardiac calcium channel with 1,4-dihydropyridine(-)-[³H]azidopine was described by Ferry et al. (1987).

Knaus et al. (1992) described a unique fluorescent phenylalkylamine probe for L-type Ca^{2+} channels.

Binding sites for ω -conotoxin appear to be primarily associated with the N-type of voltage-dependent calcium channels (Feigenbaum et al. 1988; Wagner et al. 1988).

Cohen et al. (1992) recommended the peptide ω -agatoxin IIIA as a valuable pharmacological tool being the only known ligand that blocks L-type calcium channels with high affinity at all voltages and causes, unlike the 1,4-dihydropyridines, no block of T-type calcium channels.

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A.4.2**Calcium Antagonism in Isolated Organs****A.4.2.1****Calcium Antagonism on Action Potential of Isolated Guinea Pig Papillary Muscle****PURPOSE AND RATIONALE**

Intracellular action potential in the guinea pig papillary muscle is recorded. Partial depolarization is achieved by potassium enriched Ringer solution and by addition of isoproterenol. Resting potential is increased to 40 mV resulting in inactivation of the fast sodium channel. Under these conditions, upstroke velocity is an indicator for calcium flux through the membrane, which is decreased by calcium blockers.

PROCEDURE

Guinea pigs of either sex (Pirbright White strain) weighing 300–400 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Initially, normal Ringer solution oxygenated with carbogen (95% O₂/5% CO₂) at a temperature of 36°C is used. A standard micro electrode technique is applied to measure the action potential via a glass micro electrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at intervals of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold. The intracellular action potential is amplified, differentiated for registration of upstroke velocity (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

After an incubation period of 30 min the Ringer solution is changed to the following composition containing 5 times more potassium and 10% less sodium.

- NaCl 8,1 g/L
- KCl 1,0 g/L
- CaCl₂ 0,2 g/L
- NaHCO₃ 0,1 g/L
- glucose 5,0 g/L

For further depolarization, isoproterenol (1.0 mg per 100 ml) is added. By this measure, resting potential is increased to about 40 mV, resulting in inactivation of the fast inward sodium channel. The resulting slow rising action potential is sensitive to calcium antagonistic drugs (Kohlhardt and Fleckenstein 1977).

The test compound is added at a concentration of 1 µg/ml. Effective compounds are tested at lower concentrations and compared with the standard (nifedipin at concentrations of 0.01 and 0.1 µg/ml)

EVALUATION

The decrease of upstroke velocity is tested at various concentrations of the test compound and compared with the standard.

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A.4.2.2**Calcium Antagonism in the Isolated Guinea Pig Atrium****PURPOSE AND RATIONALE**

k-Strophanthine (and other cardiac glycosides) inhibit the membrane-bound Na⁺/K⁺-activated ATP-ase which leads to an increase in intracellular Ca²⁺-concentration. Ca²⁺ ions activate the contractile apparatus, causing a distinctive enhancement of contractions. The procedure can be used to evaluate a compound's calcium channel blocking activity by measuring its ability to decrease atrial contractions induced by k-strophanthine.

PROCEDURE**Apparatus**

HSE-stimulator 1 (Hugo Sachs Elektronik, D-79232 March-Hugstetten, Germany)

Stimulation data:

- frequency 1.5 Hz
- duration 3 ms
- voltage 3–8 V

Experiment

Guinea pigs of either sex weighing 200–500 g are sacrificed with a blow to the nape of the neck and exsanguinated. The left atrium is removed, placed in an organ bath and attached to an isotonic strain gauge, its base being wired to an electrode of the stimulator. The Ringer-solution is aerated with carbogen and kept at 36°C. The atrium is continuously stimulated via stimulator 1, the voltage being slowly increased up to the threshold level. Contractions are recorded on a polygraph. Prior to drug administration, two prevalues are obtained by adding 2 µg/ml k-strophanthine- α (Cymarin) to the organ bath and measuring the increase in contractile force. Following a 15 min washout and recovery period, the test drug is added to the bath followed by administration of k-strophanthine- α 10 min later. The change in contractile force is always measured 10 min after the addition of k-strophanthine- α .

Standard compounds:

- Verapamil hydrochloride
- Nifedipine

EVALUATION

The percent inhibition of k-strophanthine- α induced contraction is determined.

MODIFICATIONS OF THE METHOD

Calcium antagonists can also be evaluated in the LANGENDORFF heart preparation (Lindner and Ruppert 1982).

Leboeuf et al. (1992) reported the protective effect of bepridil and flunarizine against veratrine-induced contracture in rat atria concluding from the results in this model that these agents may be more effective as L-type calcium ion-channel blockers in protecting against calcium overload during ischaemia and reperfusion injury.

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A.4.2.3

Calcium Antagonism in the Isolated Rabbit Aorta

PURPOSE AND RATIONALE

Contraction of aorta rings is induced by adding potassium chloride or norepinephrine to the organ bath containing slightly modified Krebs bicarbonate buffer. Test drugs with calcium channel blocking activity have a relaxing effect.

PROCEDURE

Rabbits of either sex weighing 3–4 kg are sacrificed with an overdose of pentobarbital sodium. The chest cavity is opened and the descending thoracic aorta (from the level of the aortic arch to the level of the diaphragm) is rapidly removed and placed in a beaker of oxygenated Krebs bicarbonate buffer at 37°C.

The content of magnesium and calcium is slightly diminished in the Krebs bicarbonate buffer resulting in the following composition:

- NaCl 118.4 mMol
- KCl 4.7 mMol
- KH₂PO₄ 1.2 mMol
- MgSO₄ · 2H₂O 1.2 mMol
- CaCl₂ · 2H₂O 1.9 mMol
- NaHCO₃ 25.0 mMol
- dextrose 10.0 mMol
- EDTA 0.013 mMol

The tissue is then transferred to a dish containing fresh oxygenated, warmed Krebs solution. Fat and loose connective tissue are carefully removed while keeping the tissue moist with the solution. Eight rings of 4–5 mm width are obtained and each is mounted in a 20 ml tissue bath which contains the oxygenated warmed Krebs solution. Initial tension is set at 1.0 g. The tissue is allowed to incubate over a period of 2 h, during which time the Krebs solution is changed every 15 min. Also during this time, tension is maintained at 1.0 g. Just prior to the end of the 2 h equilibration period, the Krebs solution is changed again and the tissue

is allowed to stabilize at 1.0 g tension. A sustained contraction is then generated by addition of either 40 mM KCl or 2.9×10^{-3} mM norepinephrine.

Twenty min after addition of the agonist, the test drug is added so that the final concentration in the bath is 1×10^{-5} M. The percent relaxation reading is taken 30 min after addition of the test drug. If at least 30% relaxation occurs, an accumulative concentration-relaxation curve is established. There is a 30 min period of time between the addition of each concentration of test compound.

EVALUATION

Active tension is calculated for the tissue at the time point just prior to the addition of the test compound and also at the point 30 min after the addition of each concentration of test compound. Active tension is defined as the difference between the generated tension and the baseline tension. The percent relaxation from the predrug, precontracted level is calculated for each concentration of test compound. A number of 5 experiments constitutes a dose range. An ID_{50} is calculated by linear regression analysis.

MODIFICATIONS OF THE METHOD

Hof and Vuorela (1983) compared three methods for assessing calcium antagonism on rabbit aorta smooth muscle.

Matsuo et al. (1989) reported a simple and specific screening method for Ca-entry blockers. In the presence of various Ca-channel blockers, 1×10^{-4} M Ca^{2+} causes relaxation of rat uterine smooth muscle that has been tonically contracted with oxytocin in calcium-free medium after prolonged preincubation with 3 mM EGTA.

Micheli et al. (1990) used spirally cut preparations of rat aorta and rings of rabbit ear artery to test calcium entry blocker activity.

Rüegg et al. (1985) described a smooth muscle cell line originating from fetal rat aorta to be suitable for the study of voltage sensitive calcium channels. Calcium channel antagonists inhibited both the basal and the potassium chloride stimulated $^{45}Ca^{2+}$ uptake.

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A.4.2.4

Calcium Antagonism in the Isolated Guinea Pig Pulmonary Artery

PURPOSE AND RATIONALE

Contraction of the pulmonary artery is induced by changing the normal Tyrode solution in the organ bath against a potassium enriched solution. This contraction can be inhibited by calcium blockers.

PROCEDURE

The following solutions are used:

| | Normal Tyrode solution [mMol] | Potassium Enriched Tyrode solution [mMol] |
|----------------------------------|-------------------------------|-------------------------------------------|
| NaCl | 135.0 | 89.0 |
| KCl | 3.7 | 50.0 |
| MgSO ₄ | 0.81 | 0.81 |
| NaH ₂ PO ₄ | 0.41 | 0.41 |
| NaHCO ₃ | 11.0 | 11.0 |
| CaCl ₂ | 2.25 | 2.25 |
| Glucose | 5.6 | 5.6 |

Guinea pigs (Pirbright White strain) of either sex weighing 400–500 g are sacrificed by stunning. The pulmonary artery is removed and cut spirally at an angle of 45°. The resulting strip is cut to lengths of 2 cm and one piece is suspended in oxygenated normal Tyrode solution in an organ bath at 37°C with a preload of 1 g. Contractions are registered with an isotonic strain transducer and recorded on a polygraph.

After 1 h equilibrium time, normal solution is exchanged with potassium enriched Tyrode solution. The artery strip reacts with a contraction which achieves after 10 min 90–95% of its maximum. After an additional 10 min, exchange to normal Tyrode solution is performed. Ten min later again a contraction is induced by potassium enriched solution. When the height of the contraction has reached a constant level, the test substance is added and again potassium induced contraction recorded. The height of the contraction is expressed as percent of initial potassium induced contraction.

After lavage, the procedure is repeated with a higher dose or the standard.

EVALUATION

For calculation of a regression line, the decrease of contraction versus control after various doses is measured in mm. The percentage of inhibition after various doses is taken for calculation of an ED_{50} .

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A.4.3

In Vivo Methods

A.4.3.1

Evaluation of Calcium Blockers in the Pithed Rat

PURPOSE AND RATIONALE

Using the cardioaccelerator response in pithed rats, calcium entry blockers can be distinguished from other agents which have modes of action not involving direct blockade of calcium entry (Clapham 1988).

PROCEDURE

Male Sprague-Dawley rats (250–350 g) are anaesthetized with methohexitone sodium (50 mg/kg i.p.). Following cannulation of the trachea, the rats are pithed through one orbit with a stainless steel rod and immediately artificially respired with room air (78 strokes/min, 1 ml/100 g body weight) via a Palmer

small animal respiration pump. A jugular vein is cannulated for administration of drugs. Arterial blood pressure is recorded from a carotid artery using a pressure transducer. Heart rate is derived from the phasic arterial pressure signal with a phase lock loop ratemeter (BRL Instrument Services). Both parameters are displayed on a recorder. The animals are kept warm by an incandescent lamp positioned about 25 cm above them. The pithing rod is withdrawn so that the tip lays in the thoracic portion of the spinal cord. All rats then receive (+)tubocurarine (1.5 mg/kg i.v.) and are bilaterally vagotomized.

The cardioaccelerator response is obtained by continuous electrical stimulation of the thoracic spinal cord with square wave pulses of 0.5 ms duration, at supramaximal voltage at a frequency of 0.5 Hz using the pithing rod as a stimulating electrode. An indifferent electrode is inserted subcutaneously in the femoral region. Only rats with a resulting tachycardia of more than 100 beats/min are included into the experiments.

When the cardioaccelerator response has stabilized for about 3–5 min, cumulative intravenous doses of drug or corresponding vehicle are administered. Succeding doses are given when the response to the previous dose has stabilized.

Calcium antagonists and β -blockers inhibit dose-dependent the tachycardia elicited by electrical stimulation of the spinal cord, whereas lignocaine and nicorandil are not effective.

Doses of β -blockers or calcium-antagonists, which reduce the tachycardia to 50% are tested again. Three min after administration of the drug, calcium gluconate (1 mg/min) or water (0.1 ml/min) are infused using a Harvard apparatus compact infusion pump. The effects of calcium entry blockers, but not of β -adrenoreceptor blockers, are antagonized.

EVALUATION

The level of tachycardia immediately prior to drug administration is taken as 100% and responses to drugs are expressed as a percentage of this predose tachycardia. If an inhibitory effect >50% is seen, then an ID_{50} (with 95% confidence limits) is interpolated from linear regression analysis. Significance of differences between the groups receiving calcium gluconate and their parallel vehicle controls is calculated by Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Differentiation between the effects of β -blockers and calcium-antagonists can be achieved in a relatively simple *in vivo* model.

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A.5 Anti-Arrhythmic Activity

A.5.0.1

General Considerations

Guidelines for the study of arrhythmias in man and animals regarding the experimental design as well as the classification, quantification, and analysis were given as the Lambeth Conventions by Walker et al. (1988).

Classification

Anti-arrhythmic drugs have been classified into various groups and subgroups (Vaughan Williams 1970, 1975, 1984, 1988, 1991, 1992; Borchard et al. 1989; Frumin et al. 1989; Harumi et al. 1989; Colatsky and Follmer 1990; Podrid 1990; Coromilas 1991; Nattel 1991; Rosen and Schwartz 1991; Scholz 1991; Woosley 1991; Ravens 1992; Sanguinetti 1992; Grant 1992; Nattel 1993; Scholz 1994). This classification is based on electrophysiological effects (e.g., action potential) and on interaction with membrane receptors and ion channels. The heterogeneity of classification criteria resulted in vivid discussions (The Sicilian Gambit 1991; Vaughan Williams 1991, 1992). In particular, a clinical study (CAST investigators 1990) challenged the therapeutic value of some anti-arrhythmic drugs.

Weirich and Antoni (1990, 1991) proposed a subdivision of class-I anti-arrhythmic drugs according to the saturation behavior of frequency-dependent block and its onset-kinetics.

Class I anti-arrhythmic drugs directly alter membrane conductance of cations, particularly those of Na^+ and K^+ . They reduce upstroke velocity, V_{max} , of the cardiac action potential by blockade of the fast Na^+ channel. This leads to a depression of conduction velocity, a prolongation of the voltage- and time-dependent refractory period and an increase in the threshold of excitability in cardiac muscle. Class I anti-arrhythmic drugs are subclassified according their effect on the action potential duration.

Class IA anti-arrhythmic drugs (Quinidine-like substances, e.g., disopyramide, procainamide, ajmaline) lengthen the action potential duration which is reflected in the ECG as lengthening of the QT-interval. This effect is added to that on fast sodium channel resulting in delayed recovery from inactivation.

Class IB anti-arrhythmic drugs (lidoacaine-like drugs, e.g., mexiletine, phenytoin, tocainide), in contrast, shorten the action potential duration.

Class IC anti-arrhythmic drugs (e.g., encainide, flecainide, propafenone, indecainide) produce quinidine- and lidocaine-like effects and exert differential actions on the duration of action potential in Purkinje fibres (shortening) and ventricular muscle.

Class II anti-arrhythmic drugs are β -adrenergic antagonists. They exert their anti-arrhythmic effects by antagonizing the electrophysiological effects of catecholamines which are mainly mediated by an increase in slow calcium inward current.

Class III anti-arrhythmic drugs (e.g., amiodarone, bretylium, sotalol) prolong the action potential and lead to a corresponding increase in the effective refractory period. The action is mainly due to a block of outward repolarizing currents. However, activation of sodium and calcium inward currents that prolong the plateau of the action potential may also be involved.

Class IV anti-arrhythmic drugs (e.g., verapamil, diltiazem) are slow calcium channel blockers suppressing the slow calcium inward current and calcium-dependent slow action potentials.

Experimentally Induced Arrhythmias

Winslow (1984) reviewed the methods for the detection and assessment of antiarrhythmic activity.

Szekeres (1979) suggested a rational screening program for the selection of effective antiarrhythmic drugs.

Arrhythmia models in the rat were reviewed by Cheung et al. (1993).

Arrhythmogenic stimuli can be divided into three groups: chemical, electrical and mechanical (Szekeres and Papp 1975; Wilson 1984).

Chemically Induced Arrhythmias

A large number of chemical agents alone or in combination are capable of inducing arrhythmias. Administration of anesthetics like chloroform, ether, halothane (sensitizing agents) followed by a precipitating stimulus, such as intravenous adrenaline, or cardiac glycosides (usually ouabain), aconitine, and veratrum alkaloids cause arrhythmias. The sensitivity to these arrhythmogenic substances differs among various species.

Electrically Induced Arrhythmias

The possibilities to produce arrhythmias by electrical stimulation of the heart and the difficulties for evaluation of anti-arrhythmic drugs by this approach have

been discussed by Szekeres (1971). Serial electrical stimulation result in flutter and fibrillation and it is possible to reproduce some of the main types of arrhythmias of clinical importance. The flutter threshold or the ventricular multiple response threshold may be determined in anesthetized dogs before or after the administration of the test drug.

Mechanically Induced Arrhythmias

Arrhythmias can be induced directly by ischemia or by reperfusion. After ischemia either by infarction or by coronary ligation several phases of arrhythmias are found. The two stage coronary artery ligation technique described by Harris (1950) focuses on late arrhythmias.

Curtis and Walker (1988) examined seven scores in an attempt to validate the use of arrhythmia scores in an *in vivo* model of conscious rats.

The influence on reperfusion arrhythmias can be tested in various species, e.g., rat, pig, dog and cat (Bergey et al. 1982; Winslow 1984; Curtis et al. 1987; Brooks et al. 1989).

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A.5.0.2

Electrocardiography in Animals

PURPOSE AND RATIONALE

Recording of the electrocardiogram is an essential tool in the evaluation of anti-arrhythmic drugs (Johnston et al. 1983; Curtis and Walker 1986; Adaikan et al. 1992). Similar to the heart rate, the electrocardiogram is different between various species (Bazett 1920; Kisch 1953; Heise and Kimbel 1955; Beinfeld and Lehr 1968; Budden et al. 1981; Driscoll 1981; Osborn 1981; Hayes et al. 1994). Many authors used the bipolar lead II between right foreleg and left hindleg, which is in line with the neutrally placed heart. Additionally, lead I (between right and left foreleg) stated to lie in the axis of the horizontal heart, and lead III (between left foreleg and left hindleg) in line with the vertical heart, may be used as well as unipolar leads (usually designed as V_1 to V_6) and the unipolar leads designed as aVL, aVR, and aVF. Out of several species being used the procedure for rats (Penz et al. 1992; Hayes et al. 1994) is described.

PROCEDURE

Male Sprague Dawley rats weighing 250–300 g are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbitone. The right jugular vein is cannulated for injections, while the left coronary artery is cannulated for recording blood pressure on a polygraph. The ECG is recorded using a Lead type II of configuration along the anatomical axis of the heart as determined by palpation. ECGs are recorded at a standard chart speed of 100 mm/s on a polygraph and simultaneously on a storage oscilloscope. Measurements of intervals are made on the chart recorder and from the memory trace of the monitor.

Since in the rat it is difficult to detect a T-wave that corresponds exactly with the T-wave seen in other species (Beinfeld and Lehr 1968; Driscoll 1981; Surawicz 1987) T-wave calculations are made on the basis of the repolarization wave that follows the QRS complex. The following variables are measured:

σT = time for the depolarization wave to cross the atria, *P-R* interval, *QRS* interval, *Q-T* interval, and *RSh* (the height between the peak of R and S wave). The *RSh* magnitude is taken as a measure of the extent of S-wave depression as exerted by class I sodium channel blocking antiarrhythmics.

EVALUATION

Statistical analyses are based on ANOVA followed by Duncan's test for differences of means. In order to demonstrate the relationships between and drug effects, standard cumulative dose-response curves are constructed.

MODIFICATIONS OF THE METHOD

Osborne (1973, 1981) described a restraining device facilitating electrocardiogram recording in **conscious rats**.

Curtis and Walker (1986), Johnston et al. (1983) studied the responses to ligation of a coronary artery and the actions of antiarrhythmics in conscious rats.

Hayes et al. (1994) studied the ECG in **guinea pigs**, rabbits and primates.

Stark et al. (1989) described an epicardic surface and stimulation technique (SST-ECG) in Langendorff perfused guinea pig hearts.

Epicardial His bundle recordings in the guinea pig *in vivo* were described by Todt and Raberger (1992).

Chronic recording from the His bundle in awake nonsedated **dogs** was reported by Karpawich et al. (1983) and by Atlee et al. (1984).

Van de Water et al. (1989) reported a formula to correct the QT interval of the electrocardiogram in dogs for changes in heart rate.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-reentry and ventricular antifibrillatory studies in anesthetized dogs. The reentry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The pacing induced tachycardia that results from circus movements around the tricuspid ring was very persistent in duration and regular in cycle length. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

Weissenburger et al. (1991) developed an experimental model of the long QT syndrome in conscious dogs for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of “torsades de pointes.”

Bauer et al. (2004) described pro- and antiarrhythmic effects of fast cardiac pacing in a canine model of acquired long QT syndrome.

Holter monitoring in conscious dogs was described by Krumpl et al. (1989a, b).

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Baboons and **monkeys** (*Macaca sp.*) were used by Adaikan et al. (1992).

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A.5.0.3

Aconitine Antagonism in Rats

PURPOSE AND RATIONALE

The plant alkaloid aconitine persistently activates sodium channels. Infusion of aconitine in the anesthetized rat causes ventricular arrhythmias. Drugs considered to have anti-arrhythmic properties can be tested in aconitine-intoxicated rats.

PROCEDURE

Male Ivanovas rats weighing 300–400 g are used. The animals are anesthetized by intraperitoneal injection of 1.25 g/kg urethane. Five $\mu\text{g}/\text{kg}$ aconitine dissolved in 0.1 N HNO_3 is administered by continuous infusion into the saphenous vein of 0.1 ml/min and the ECG in lead II is recorded every 30 seconds. The test compound is injected orally or intravenously at a screening dose of 3 mg/kg 5 min before the start of the aconitine infusion. Eight–ten animals are used per compound.

EVALUATION

The anti-arrhythmic effect of a test compound is measured by the amount of aconitine/100g animal (duration of infusion) which induces

- ventricular extrasystoles
- ventricular tachycardia
- ventricular fibrillation
- and death.

Higher doses of aconitine in the treated group as compared to an untreated control group are an indication of anti-arrhythmic activity.

Statistical significance between the groups is assessed by the Student's *t*-test.

The scores are allotted for the intensity and the duration of the effect relative to the efficacy of standard compounds.

Standard data:

- Procainamid, 5 mg/kg i.v. and lidocaine, 5 mg/kg, i.v. lead to an increase in LD_{100} by 65% (corresponds to LD_{100} of approximately 9 μ g/100 g).

CRITICAL ASSESSMENT OF THE METHOD

Aconitine – antagonism *in vivo* has been proven as a valuable screening method for anti-arrhythmic activity.

MODIFICATIONS OF THE METHOD

Scherf (1947) studied the auricular tachycardia caused by aconitine administration in **dogs**.

Scherf et al. (1960) provoked atrial flutter and fibrillation in anesthetized dogs by application of a few crystals of aconitine or delphinine to the surface of the right atrium in the appendix area near the head of the sinus node.

McLeod and Reynold (1962) induced arrhythmia by aconitine in the isolated **rabbit** atrium.

Nwangwu et al. (1977) used aconitine as arrhythmogenic agent for screening of anti-arrhythmic agents in **mice**.

Yamamoto et al. (1993) used urethane-anesthetized rats under artificial respiration with tubocurarine pretreatment. After thoracotomy and incision of the pericardium, a piece of filter paper soaked with aconitine solution was applied to the right atrium. Test drugs were applied by continuous i.v. infusion. In addition to ECG lead II, intra-atrial ECG was monitored.

Aconitine-antagonism in conscious mice as screening procedure has been recommended by Dadkar and Bhattacharya (1974) and in anesthetized mice by Winslow (1980).

Nakayama et al. (1971) described the topical application of aconitine in a small cup placed on the right atrium of dogs to induce supraventricular arrhythmias.

A method using the **cat** has been developed by Winslow (1981).

Other Arrhythmogenic Agents

In addition to the aconitine model Vaillie et al. (1992) demonstrated the selectivity of a $CaCl_2$ continuous infusion screening method in rats for the evaluation of antiarrhythmic calcium antagonists.

A mouse chloroform model was recommended by Lawson (1968).

Vargaftig et al. (1969) induced ventricular fibrillation in mice by inhalation of chloroform.

Papp et al. (1967) proposed the experimental $BaCl_2$ -arrhythmia as a quantitative assay of anti-arrhythmic drugs.

Al-Obaid et al. (1998) used calcium chloride-induced arrhythmias for anti-arrhythmic activity evaluation in anesthetized male rats. Cardiac arrhythmias were induced by a single intravenous injection of 10% $CaCl_2$ (50 mg/kg). The induced arrhythmias were then analyzed for magnitude of initial bradycardia, onset, incidence and duration of the induced fibrillations. After the induction of the arrhythmia, the animal was allowed to recover completely (15–20 min) and the test compound was injected in different doses intravenously. The effect of the test compound on the basal heart rate was then examined and the percentage change in the heart rate was calculated. Seven min later, the arrhythmogenic dose of $CaCl_2$ was readministered and the effect of the treatment on the induced arrhythmia parameters was evaluated as percentage change in the measured parameters or as protection or non-protections against the induced fibrillations.

Tripathi and Thomas (1986) described a method for the production of ventricular tachycardia in the rat and guinea pig by exposing the animals to benzene vapors for 2 min followed by an intravenous adrenaline injection.

Arrhythmias could be induced by changing the medium of cultured rat heart muscle cells (Wenzel and Kloeppel 1978).

In isolated rat hearts ventricular fibrillation was induced by isoprenaline and a catechol-O-methyl transferase inhibitor at high perfusion temperature (Sono et al. 1985).

Takei (1994) described experimental arrhythmia in guinea pigs induced by grayanotoxin-I, a biologically active diterpenoid from the plant family of Ericaceae.

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A.5.0.4

Digoxin-Induced Ventricular Arrhythmias in Anesthetized Guinea Pigs

PURPOSE AND RATIONALE

Overdose of cardiac glycosides, such as digoxin, induces ventricular extrasystoles, ventricular fibrillation, and finally death. The occurrence of these symptoms can be delayed by anti-arrhythmic drugs.

PROCEDURE

Male guinea pigs (Marioth strain) weighing 350–500 g are anesthetized with 35 mg/kg pentobarbital sodium intraperitoneally. Trachea, one jugular vein and one carotid artery are catheterized. Positive pressure ventilation is applied with a respiratory pump (Rhema GmbH, Germany) at 45 breaths/min. The carotid artery is used for monitoring systemic blood pressure via a pressure transducer. Digoxin is infused into the jugular vein with a perfusion pump (ASID BONZ PP 50) at a rate of 85 $\mu\text{g}/\text{kg}$ in 0.266 ml/min until cardiac arrest. The electrocardiogram (lead III) is recorded with subcutaneous steel-needle electrodes (Hellige 19).

Treated groups ($n = 5\text{--}10$ animals) receive the test drug either orally 1 h or intravenously 1 min prior to the infusion. The control group ($n =$ at least 5 animals) receives the digoxin infusion only. The period until the onset of ventricular extrasystoles, ventricular fibrillation, and cardiac arrest is recorded. The total amount of infused digoxin ($\mu\text{g}/\text{kg}$) to induce ventricular fibrillation is calculated. Standard drugs are lidocaine (3 mg/kg i.v.) or ramipril (1 mg/kg p.o.).

EVALUATION

Using Student's *t*-test the doses of digoxin needed to induce ventricular extrasystoles, or ventricular fibrillation, or cardiac arrest, respectively, after treatment with anti-arrhythmic drugs are compared statistically with controls receiving digoxin only.

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A.5.0.5

Strophanthin or Ouabain Induced Arrhythmia

PURPOSE AND RATIONALE

Acute intoxication with the cardiac glycoside strophanthin K induces ventricular tachycardia and multifocal ventricular arrhythmias in dogs. This can be used as a test model to evaluate the effect of potential antiarrhythmic drugs on ventricular arrhythmias.

PROCEDURE

Male or female dogs of either sex weighing approximately 20 kg are used. The animals are anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium. Two peripheral veins are cannulated for the administration of the arrhythmia-inducing substance (*V. brachialis*) and the test compound (*V. cephalica antebrachii*). For intraduodenal administration of the test drug, the duodenum is cannulated. Electrocardiogram is registered with needle electrodes from lead II. Heart frequency is derived from R-peaks of ECG. Two–three animals are used for one compound.

Strophanthin K is administered by continuous i.v.-infusion at a rate of 3 µg/kg/min. Thirty–fourty min later, signs of cardiac glycoside intoxication appear leading to ventricular tachycardia or to multifocal ventricular arrhythmias. When this state is achieved, the strophanthin infusion is terminated. When the arrhythmias are stable for 10 min, the test substance is administered intravenously in doses between 1.0 and 5.0 mg/kg or intraduodenally in doses between 10 and 30 mg/kg.

ECG II recordings are obtained at times: -0.5, 1, 2, 5 and 10 min following administration of test drug.

For i.v. administration: A test compound is considered to have an antiarrhythmic effect if the extrasystoles immediately disappear. If the test compound does not show a positive effect, increasing doses are administered at 15 min-intervals. If the test substance does

reverse arrhythmias, the next dose is administered after the reappearance of stable arrhythmias.

For i.d. administration: A test compound is considered to have a definite antiarrhythmic effect if the extrasystoles disappear within 15 min. The test drug is considered to have “no effect” if it does not improve strophanthin intoxication within 60 min following drug administration.

EVALUATION

Evaluation of the therapeutic effect of a drug is difficult and somewhat arbitrary since there is no clear-cut correlation between effectiveness of a test compound and duration of its effect, i. e. return to normal ECGs. The standard drugs ajmaline, quinidine and lidocaine re-establish normal sinus rhythm at doses of 1 and 3 mg/kg (i.v.) and 10 mg/kg (i.d.). Arrhythmias are eliminated for 20 min (i.v.) and for > 60 min (i.d.) following drug administration.

MODIFICATIONS OF THE METHOD

Ettinger et al. (1969) used arrhythmias in dogs induced by ouabain to study the effects of phentolamine in arrhythmia.

Garrett et al. (1964) studied the antiarrhythmic activity of *N,N*-diisopropyl-*N'*-diethylaminoethylurea hydrochloride in anesthetized dogs with arrhythmias induced by ouabain, aconitine or acetylcholine. Furthermore, ultra-low frequency ballistocardiograms with ECG registration were performed in dogs.

Raper and Wale (1968) studied the effects on ouabain- and adrenaline-induced arrhythmias in **cats**.

Kerr et al. (1985) studied the effects of a vasodilator drug on ouabain-induced arrhythmias in anesthetized dogs.

A modified method for the production of cardiac arrhythmias by ouabain in anesthetized cats was published by Rao et al. (1988).

Brooks et al. (1989) infused ouabain intravenously to **guinea pigs** and determined the onset of ventricular extrasystoles and of fibrillation.

Thomas and Tripathi (1986) studied the effects of α -adrenoreceptor agonists and antagonists with different affinity for α_1 - and α_2 -receptors on ouabain-induced arrhythmias and cardiac arrest in guinea pigs.

Krzeminski (1991) and Wascher et al. (1991) used ouabain-induced arrhythmia in guinea pigs for the evaluation of potential antidysrhythmic agents.

Al-Obaid et al. (1998) used ouabain-induced arrhythmias in anesthetized **Wistar rats** for evaluation of cyclopenteno[b]thiophene derivatives as antiarrhythmic agents.

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A.5.0.6

Ventricular Fibrillation Electrical Threshold

PURPOSE AND RATIONALE

The use of anti-arrhythmic drugs in the treatment of ventricular arrhythmias aims to prevent the development of ventricular fibrillation. Several electrical stimulation techniques have been used to measure ventricular fibrillation threshold such as single pulse stimulation, train of pulses stimulation, continuous 50-Hz stimulation and sequential pulse stimulation.

PROCEDURE

Adult dogs weighing 8–12 kg are anesthetized with sodium pentobarbital (35 mg/kg) and ventilated with air using a Harvard respiratory pump. Systolic arte-

rial pressure is monitored and body temperature maintained by a thermal blanket. The chest is opened by a midline sternotomy and the heart suspended in a pericardial cradle. The sinus node is crushed and a 2.0 mm diameter Ag-AgCl stimulating electrode is embedded in a Teflon disc sutured to the anterior surface of the left ventricle. The heart is then driven by 3-ms square anodal constant current pulses for 400 ms of the basic cycle and is prematurely stimulated by one 3-ms test stimulus through the driving electrode. Electrical stimulation is programmed by a digital stimulator. A recording electrode is placed on the surface of each ventricle. A silver plate is implanted under the skin in the right femoral region as indifferent electrode. Lead II of the body surface electrocardiogram is monitored. To determine ventricular fibrillation threshold (VFT), a 0.2- to 1.8-second train of 50-Hz pulses is delivered 100 ms after every eighteenth basic driving stimulus. The current intensity is increased from the diastolic threshold in increments of 10 μ A to 1.0 mA or until ventricular fibrillation occurs. The minimal current intensity of the pulse train required to induce sustained ventricular fibrillation is defined as the VFT. When ventricular fibrillation occurs, the heart is immediately defibrillated and allowed to recover to control conditions for 15 to 20 min. Anti-arrhythmic drugs are administered through the femoral vein.

EVALUATION

Ventricular fibrillation threshold (VFT) is determined before and after administration of test drugs at given time intervals. The mean values of 10 experiments are compared using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Marshall et al. (1981) and Winslow (1984) suggested to determine VFT in the pentobarbitone anesthetized rat.

Wu et al. (1989) recommended a conscious dog model for re-entrant atrial tachycardia.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-re-entry and ventricular antifibrillatory studies in dogs. The re-entry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

A chronically prepared rat model of electrically induced arrhythmias was described by Walker and Beach (1988).

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A.5.0.7**Coronary Artery Ligation, Reperfusion Arrhythmia and Infarct Size in Rats****PURPOSE AND RATIONALE**

Coronary artery ligation in anesthetized rats results in arrhythmias and myocardial infarction. Following occlusion of the left main coronary artery, very marked ventricular dysrhythmias occur. Electrocardiogram is recorded during ligation and subsequent reperfusion. The amount of infarcted tissue is measured by means of p-nitro-blue tetrazolium chloride-staining in myocardial sections. The model is used to test drugs with potential anti-arrhythmic activities.

PROCEDURE

Groups of 8–10 male Sprague-Dawley rats weighing 350–400 g are used. The animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. The trachea is intubated to allow artificial ventilation (Starling pump). A catheter is placed in an external jugular vein for administration of test com-

pounds. Peripheral blood pressure is recorded from the common carotid artery using a pressure transducer and a polygraph. The chest is opened by left thoracotomy at the fourth intercostal space. After opening the pericard, the heart is exteriorized by gentle pressure on the chest walls and a thin silk thread (Ethicon 1.5 metric, 4–0) attached to an atraumatic needle is placed around the left coronary artery about 2–3 mm distal of the origin of the left coronary artery for later ligation. From that point on, the animal is ventilated with room air using a stroke volume of 1 ml/100 g body weight at a rate of 54 strokes/min. The heart is then placed back in the chest cavity. Any animal in which this procedure itself produces dysrhythmias or a sustained fall in mean arterial blood pressure to less than 70 mm Hg has to be discarded from the study.

After an equilibration time of approx. 45 min, the test substance or the vehicle (control) is administered by intravenous injection. Five min later, the ligature at the left coronary artery is closed either for 15 or 90 min (in case infarct-size is assessed) and subsequently reperfused for 30 min. For oral application, the test compounds are dissolved or suspended in the vehicle 30 min before occlusion. Peripheral blood pressure and ECG lead II are recorded continuously during the whole experiment. Rectal temperature is maintained at 38°C. The numbers of ventricular premature beats (VPB), ventricular tachycardia (VT) and ventricular fibrillation (VF) are counted in the occlusion and reperfusion periods and evaluated according to the guidelines of the Lambeth Convention (Walker et al. 1988).

Preparation to Determine Infarct Size

At the end of the reperfusion period, the animal is sacrificed with an overdose of pentobarbital sodium, the heart is dissected and cut into transversal sections (approx. 1 mm thick) from the apex to the base. The slices are stained with p-nitro-blue tetrazolium chloride solution (0.25 g/L p-nitro-blue tetrazolium chloride in Sørensen phosphate buffer, containing 100 mM D, L-maleate) in order to visualize the infarct tissue (blue/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of infarct area. Left ventricle and infarct area are measured by planimetry from projections of all slices with the exclusion of the apex and the slice containing the ligature.

EVALUATION

The following parameters are evaluated:

- mortality
- hemodynamics
 - peripheral blood pressure [mm Hg]
 - heart rate [beats/min]
 - pressure rate index (PRI) (BPs × HR) [mm Hg × beats/1000]
- arrhythmias
 - ventricular extrasystoles (= premature ventricular contractions) (PVC)
 - percent animals with PVC
 - number of PVC/5 or 30 min
 - ventricular tachycardia (VT) (VT defined as any run of seven or more consecutive ventricular extrasystoles)
 - percent animals with VT
 - duration [s] of VT/5 or 30 min
 - ventricular fibrillation (VF)
 - percent animals with VF
 - duration [s] of VF/5 or 30 min
- infarct size (area)

The different characteristics are evaluated separately and compared with a positive control (5 mg/kg nicaïnoprol i.v.).

Changes of parameters in drug-treated animals are compared to vehicle control values.

Statistical significance is assessed by the Student's *t*-test.

MODIFICATIONS OF THE METHOD

Leprán et al. (1983) placed a loose silk loop around the left coronary artery and passed the thread through a cylinder shaped polyethylene tube outside the thorax. The rats were allowed to recover from primary surgery. The loose ligature was tightened 7–10 days thereafter and arrhythmias recorded by ECG tracings.

Johnston et al. (1983) described the responses to ligation of a coronary artery in conscious rats and the actions of anti-arrhythmics.

As reported in Sect. A.3.1.2 and A.3.1.3, the isolated heart according to LANGENDORFF and the isolated working rat heart preparation can be used for ligation experiments inducing arrhythmias. Lubbe et al. (1978) reported ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart as a model for assessment of anti-fibrillatory action of anti-arrhythmic agents.

Bernier et al. (1986) described reperfusion-induced arrhythmias in the isolated perfused rat heart. The isolated rat heart was perfused according the LANGENDORFF-technique. A ligature was placed around the left anterior descending coronary artery close to its ori-

gin. The arterial occlusion was maintained for 10 min followed by reperfusion. Test compounds were included in the perfusion medium. With epicardial ECG-electrodes the number of premature ventricular complexes, the incidence and duration of ventricular fibrillation, and the incidence of ventricular tachycardia were recorded.

Abraham et al. (1989) tested antiarrhythmic properties of tetrodotoxin against occlusion-induced arrhythmias produced by ligation of the left anterior descending coronary artery in the rat.

MacLeod et al. (1989) tested a long acting analogue of verapamil for its actions against arrhythmias induced by ischemia and reperfusion in conscious and anesthetized rats, as well as for effects on epicardial intracellular action potentials.

Harper et al. (1993) found that the inhibition of Na⁺/H⁺ exchange preserves viability, restores mechanical function, and prevents the pH paradox in reperfusion injury to rat neonatal myocytes.

Scholz et al. (1993, 1995) reported protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion in rats.

Likewise, Yasutake et al. (1994) found protection against reperfusion-induced arrhythmias in rats by intracoronary infusion of a Na⁺/H⁺ exchange inhibitor.

Aye et al. (1997) tested the effects of a Na⁺/H⁺ exchange inhibitor on reperfusion ventricular arrhythmias in rat hearts.

Ferrara et al. (1990) studied the effect of flecainide acetate on reperfusion- and barium-induced ventricular tachyarrhythmias in the isolated perfused rat heart by monitoring heart rate, coronary flow rate, left ventricular systolic pressure, dp/dt_{max} , and the voltage of the epicardial electrogram.

Heterogeneity of ventricular remodeling after acute myocardial infarction in rats has been reported by Cappasso et al. (1992).

Bellemin-Baurreau et al. (1994) described an *in vitro* method for evaluation of antiarrhythmic and anti-ischemic agents by using programmed electrical stimulation of the isolated rabbit heart after ligation of the left ventricular branch of the coronary artery and a reperfusion period of 15 min.

The use of the rat in models for the study of arrhythmias in myocardial ischemia and infarction has been reviewed by Curtis et al. (1987).

Black and Rodger (1996), Black (2000) reviewed the methods used to study experimental myocardial ischemic and reperfusion injury in various animal species.

Linz et al. (1997) reported that in isolated rat hearts with ischemia-reperfusion injuries, perfusion with bradykinin reduces the duration and incidence of ventricular fibrillations, improves cardiodynamics, reduces release of cytosolic enzyme, and preserves energy-rich phosphate and glycogen stores.

Mulder et al. (1998) studied the effects of chronic treatment with calcium antagonists in rats with chronic heart failure induced by coronary artery ligation.

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A.5.0.8

Ventricular Arrhythmia After Coronary Occlusion

A.5.0.8.1

Ventricular Fibrillation After Coronary Occlusion and Reperfusion in Anesthetized Dogs

PURPOSE AND RATIONALE

Coronary artery occlusion in anesthetized dogs is accompanied by an increase in heart rate, heart contractility, left ventricular end-diastolic pressure, and blood pressure as well as by ventricular arrhythmias. During a subsequent reperfusion period, a high percentage of control animals die from ventricular fibrillation. Drugs with potential protective effects are tested which reduce both hemodynamic and electrical changes.

PROCEDURE

Dogs of either sex weighing 20–25 kg are used. Anesthesia is induced by intravenous injection of 30 mg/kg thiobutobarbital sodium and maintained by i.v. administration of 20 mg/kg chloralose and 250 mg/kg urethane followed by subcutaneous administration of 2 mg/kg morphine. The animals are placed

in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator (Bird Mark 7). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously in lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral artery is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular end-diastolic pressure (LVEDP) is measured on a high-sensitivity scale; heart rate (HR) is determined from the LVP wave form. Myocardial contractility is measured as the rate of rise of LVP (dp/dt max). The sum of ST-segment elevations is calculated from five values of the peripheral limbs in ECG lead II. The pressure-rate index ($PRI = BPs \times HR$) serves as a measure of oxygen consumption.

Experimental Course

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left anterior descending coronary artery (LAD) is prepared. A silk suture is placed around the LAD, just below the first diagonal branch. After an equilibration period of approx. 45 min., the test substance or the vehicle (controls) is administered as an intravenous bolus. Twenty min later, the ligation at the coronary artery is closed for 90 min. During the occlusion period, the test compound or the vehicle (controls) are given by continuous infusion. After release of the coronary obstruction, the animal is monitored for a 30 min reperfusion period. All parameters are recorded during the whole experiment. At the end of the test, surviving animals are sacrificed by an overdose of pentobarbital sodium.

EVALUATION

The following parameters are evaluated:

- mortality
- hemodynamics
- arrhythmias
 - ventricular extrasystoles (=premature ventricular contractions) (PVC)
 - percent animals with PVC
 - number of PVC/5 or 30 min

- ventricular tachycardia (VT) (VT defined as any sequence of seven or more consecutive ventricular extrasystoles)
- duration [s] of VT/5 or 30 min
- ventricular fibrillation (VF)
- percent animals with VF

The different characteristics are evaluated separately. Changes of parameters in drug-treated animals are compared to vehicle controls. Statistical significance of the differences is calculated by means of the Student's *t*-test.

Standard data:

Mortality: In an representative experiment, 10 out of 12 of control animals died from ventricular fibrillation during the 30 min reperfusion period. One out of 8 molsidomine-treated animals died and the death was also from ventricular fibrillation during the reperfusion phase. (Molsidomine was given as a continuous infusion of 0.5 mg/kg/ml/min during the occlusion period; controls received saline).

MODIFICATIONS OF THE METHOD

Varma and Melville (1963) described ventricular fibrillation induced by coronary occlusion during hypothermia in **dogs**.

Wilkerson and Downey (1978) described a technique for producing ventricular arrhythmias in dogs through coronary occlusion by an embolus (glass beads) being introduced into the coronary circulation via a rigid cannula which is inserted through the carotid artery.

Weissenburger et al. (1991) described a model in dogs suitable for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes".

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Thiemermann et al. (1989) described a rabbit model of experimental myocardial ischemia and reperfusion. Drugs were administered by intravenous infusion 5 min after the occlusion of the left anterior-lateral coronary artery and continued during the 60 min occlusion and subsequent 3 h reperfusion periods.

Hendriks et al. (1994) reported that the Na⁺-H⁺ exchange inhibitor HOE 694 improves post-ischemic function and high-energy phosphate resynthesis and reduced Ca²⁺ overload in the isolated perfused rabbit heart.

Barrett et al. (1997) described a method of recording epicardial monophasic action potentials and is-

chemia-induced arrhythmias following coronary artery ligation in intact rabbits.

Naslund et al. (1992) described a closed chest model in **pigs**. Occlusion was induced in pentobarbitone anesthetized, mechanically ventilated **pigs** by injection of a 2 mm ball into a preselected coronary artery. Reperfusion was achieved by retraction of the ball via an attached filament.

D'Alonzo et al. (1994) evaluated the effects of potassium channel openers on pacing- and ischemia-induced ventricular fibrillation in anesthetized **pigs**.

Sack et al. (1994) described the effects of a Na⁺/H⁺ antiporter inhibitor on post-ischemic reperfusion in pig heart.

Premaratne et al. (1995) used a **baboon** open chest model of myocardial ischemia and reperfusion. Baboons underwent occlusion of the left anterior descending coronary artery for 2 h. Fifteen min after occlusion, the treated group received hyaluronidase i.v. over a 10-min period. The ischemic period was followed by 22 h of reperfusion. At the end of the reperfusion period, the hearts were excised and the perfusion bed at risk for infarction was determined by infusion of a microvascular dye.

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A.5.0.8.2

Harris Dog Model of Ventricular Tachycardia

PURPOSE AND RATIONALE

In 1950, Harris found that the mortality in dogs after coronary occlusion with a 2-stage ligation procedure was lower than with 1-stage ligation. The left descending coronary artery is partially occluded for 30 min after which time total ligation is performed. Under these conditions arrhythmias develop within 4–7 h, reach a peak between 24 and 48 h and abate within 3–5 days.

PROCEDURE

Surgical Procedure

Dogs of either sex are anesthetized by intravenous injection of methohexitone sodium (10 mg/kg), an endotracheal tube is inserted, and anesthesia maintained with halothane. The heart is exposed through an incision in the fourth or fifth intercostal space. The anterior descending branch of the left coronary artery is dissected free below its second branch and ligated in two

stages. Two ligatures are placed around the artery and a 21 gauge needle. The first ligature is tied round the artery and the needle, which is then removed. Thirty min later, the second ligature is tied tightly round the artery. The chest is closed in layers 30 min after the second ligature has been tied, and the dog is allowed to recover.

Test Procedure

Further observations are made when the dogs are conscious, e.g., 22–24 h after ligation of the coronary artery. The dogs are positioned to lie on their side and remain in this position throughout the experiment. Mean blood pressure is recorded from a catheter placed in the femoral artery. Lead II and aV_L of the electrocardiogram and blood pressure are continuously recorded for a control period of 30 min before and during drug administration. Drugs are administered either by injection or by continuous infusion via a hind leg vein.

EVALUATION

The number of sinus and ectopic beats are counted for each successive 5-min period. Beats with a distinct P wave preceding a mean frontal QRS vector of normal duration are counted as sinus in origin; all others are denoted as ectopic.

MODIFICATIONS OF THE METHOD

The model which resembles late arrhythmias occurring in postinfarction patients has been used with modifications by many authors (e.g., Kerr et al. 1985; Reynolds and Brown 1986; Gomoll 1987; Garthwaite et al. 1989; Krumpl et al. 1989a, b; Trolese-Mongheal et al. 1985, 1991; Spinelli et al. 1991).

Methods for producing experimental complete atrioventricular block in dogs were described and reviewed by Dubray et al. (1983) and by Boucher and Duchene-Marullaz (1985).

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A.5.0.8.3

Protection against Sudden Coronary Death

PURPOSE AND RATIONALE

The group of Lucchesi described an experimental dog model to test protection against sudden coronary death (Patterson et al. 1982; Uprichard et al. 1989a, b; Chi et al. 1990a, b, 1991; Kitzen et al. 1990; Black et al. 1991, 1993).

Surgical Preparation

Purpose-bred male mongrel dogs weighing 14–22 kg are anesthetized with 30 mg/kg pentobarbital i.v. The dogs are ventilated with room air through a cuffed endotracheal tube and a Harvard respirator. A cannula is inserted in the left external jugular vein. A left thoracotomy is performed between the fourth and fifth ribs, and the heart is exposed and suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) is isolated at the tip of the left atrial appendage, and the left circumflex coronary artery (LCX) is iso-

lated ~1 cm from its origin. After a 20-gauge hypodermic needle has been placed on the LAD, a ligature is tied around the artery and the needle. The needle is then removed, resulting in critical stenosis of the vessel. The LAD is perfused for 5 min in the presence of the critical stenosis. Ischemic injury of the anterior ventricular myocardium is achieved by 2-h occlusion of the LAD by a silicon rubber snare. The vessel is reperfused after 2 h in the presence of the critical stenosis. During the period of LAD reperfusion, an epicardial bipolar electrode (1-mm silver posts, 3-mm interelectrode separation) is sutured on the left atrial appendage for subsequent atrial pacing. A bipolar plunge electrode (25-gauge stainless steel, 5 mm long, 3 mm separation) is sutured on the interventricular septum, adjacent to the occlusion site and overlying the right ventricular outflow tract (RVOT). Two similar stainless steel bipolar plunge electrodes are sutured to the left ventricular (LV) wall: one at the distribution of the LAD distal to the occlusion (infarct zone, IZ), and the second in the distribution of the LCX (non-infarct zone, NZ). A 30-gauge silver-coated copper wire electrode is passed through the wall and into the lumen of the LCX and sutured to the adjacent surface of the heart. Silver disc electrodes are implanted subcutaneously for ECG monitoring. The surgical incision is closed and the animals are allowed to recover.

Drug Treatment

The animals are treated after the recovery period during the 3 days of programmed electrical stimulation either with the test drug or with the solvent.

Electrophysiologic Studies and Programmed Electrical Stimulation

Programmed electrical stimulation (PES) is performed between days 3 and 5 after induction of anterior myocardial infarction by occlusion/perfusion of the LAD. Animals are studied while conscious and unsedated. Heart rate, ECG intervals and other electrophysiologic parameters (for details see original publications) are determined before PES is started. Premature ventricular stimuli are introduced in the region of the right ventricular outflow tract. The extra stimuli are triggered from the R-wave of the ECG, and the R-S₂ coupling interval is decreased from 350 ms until ventricular refractoriness occurs. At this time, double and triple ventricular extra stimuli are introduced during sinus rhythm. Ventricular tachyarrhythmias are defined as ‘non-sustained’, if five or more repetitive ventricular responses are initiated reproducibly, but terminated spontaneously. Ventricular tachyarrhythmias are

defined as 'sustained', if they persist for at least 30 s or, in the event of hemodynamic compromise, require ventricular burst pacing for their termination.

Sudden Cardiac Death

A direct anodal 15 μ A current from a 9-V nickel-cadmium battery is passed through a 250 Ohm resistor and applied to the electrode in the lumen of the left circumflex coronary artery. The cathode of the battery is connected to a s.c. implanted disc electrode. Lead II ECG is recorded for 30 s every 15 min on a cardiocassette recorder. After 24 h of constant anodal current or development of ventricular fibrillation, the animals are sacrificed, the hearts are excised and the thrombus mass in the LCX is removed and weighed. The heart is sectioned transversely and incubated for 15 min at 37°C in a 0.4% solution of tetrazolium triphenyl chloride for identification of infarcted areas. Time of onset of ventricular ectopy and of lethal arrhythmia is provided from recordings of the cardiocassette.

EVALUATION

Non-sustained and sustained tachyarrhythmias are evaluated.

CRITICAL ASSESSMENT OF THE METHOD

Sudden coronary death is one of the leading causes of death in developed countries. These facts warrant the use of complicated models in higher animals for search of active drugs.

MODIFICATIONS OF THE METHOD

Schwartz et al. (1984) described an experimental preparation for sudden cardiac death in dogs. The animals were chronically instrumented and studied 1 month after an anterior myocardial infarction. A balloon catheter around the circumflex coronary artery was inflated to produce acute myocardial ischemia and the occlusion was maintained for 2 min. Several days later, the animals were subjected to a submaximal stress on a motor-driven treadmill for 12–18 min. During the last minute of exercise the left coronary artery was occluded, the treadmill stopped, and the occlusion was maintained for a second minute.

Schwartz et al. (1988) analyzed the baroreceptor reflexes in conscious dogs with and without a myocardial infarction to get insights in the mechanisms of sudden death.

Cahn and Cervoni (1990) reviewed of the use of animal models of sudden cardiac death for drug development.

Pak et al. (1997) found that canine tachycardia-induced cardiomyopathy is a useful model for studying mechanisms and therapy of sudden cardiac death in heart failure. Adamson et al. (1994) performed a longitudinal study in dogs at high and low risk for sudden death and found an unexpected interaction between β -adrenergic blockade and heart rate variability before and after myocardial infarction.

Basso et al. (2004) recommended arrhythmogenic right ventriculopathy causing sudden death in boxer dogs as an animal model of human disease.

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A.5.0.8.4

Ventricular Fibrillation Induced by Cardiac Ischemia During Exercise

PURPOSE AND RATIONALE

Billman and his group developed methods to evaluate antiarrhythmic drugs for their activity in cardiovascular parameters in an exercise-plus-ischemia test.

PROCEDURE

Surgical Preparation

Mongrel dogs, weighing 15.4 to 19.1 kg, are anesthetized and instrumented to measure left circumflex CBF, left ventricular pressure and ventricular electrogram (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984). The animals are given Innovar Vet (0.02 mg/kg fentanyl citrate and 1 mg/kg hydroperidol i.v.) as a preanesthetic, whereas a surgical plane of anesthesia is induced with sodium pentobarbital (10 mg/kg i.v.). A left thoracotomy is made in the fourth intercostal space, and the heart is exposed and supported by a pericardial cradle. A 20-MHz pulsed Doppler flow transducer and a hydraulic occluder are placed around the left circumflex artery. A pair of insulated silver-coated wires are sutured to the epicardial surface of both the left and right ventricles. These electrodes are used for ventricular pacing or to record a ventricular electrogram from which HR is determined using a Gould Biotachometer (Gould Instruments, Cleveland, OH). A precalibrated solid-state pressure transducer (Konigsberg Instruments, Pasadena, CA) is inserted into the left ventricle via a stab wound in the apical dimple. Finally, a two-stage occlusion of the left anterior descending coronary artery is performed approximately one third the distance from the origin to induce an anterior wall myocardial infarction. This vessel is partially occluded for 20 min and then tied off. All leads from the cardiovascular instrumentation are tunneled under the skin

to exit on the back of the animal's neck. A transdermal fentanyl patch that delivers 75 µg/h for 72 h is placed on the back of the neck (secured with adhesive tape) to decrease postoperative discomfort. In addition, bupivacaine HCl, a long-acting local anesthetic, is injected to block the intercostal nerves (i. e., pain fibers) in the area of the incision. Each animal is placed on prophylactic antibiotic therapy (amoxicillin 500 mg p.o.) three times daily for 7 days. The animals are placed in an "intensive care" setting for the first 24 h and placed on antiarrhythmic therapy (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984).

Exercise-Plus-Ischemia Test

The studies begin 3 to 4 weeks after the production of the myocardial infarction. The animals are walked on a motor-driven treadmill and trained to lie quietly without restraint on a laboratory table during this recovery period. Susceptibility to VF is then tested. The animals run on a motor-driven treadmill while workload is increased every 3 min for a total of 18 min. The protocol begins with a 3-min warm-up period, during which the animals run at 4.8 km/h at 0% grade. The speed is increased to 6.4 km/h, and the grade is increased every 3 min as follows: 0%, 4%, 8%, 12% and 16%. During the last minute of exercise, the left circumflex coronary artery is occluded, the treadmill is stopped and the occlusion is maintained for 1 additional min (total occlusion time, 2 min). Large metal plates (diameter, 11 cm) are placed across the animal's chest so that electrical defibrillation can be achieved with minimal delay but only after the animal is unconscious (10–20 s after VF begin). The occlusion is immediately released if VF occur.

The animals then receive one or more of the following treatments:

- 1 the exercise-plus-ischemia test is repeated after pretreatment with the standard drug glibenclamide (1.0 mg/kg i.v.). The drug is injected in a cephalic vein; 3 min before exercise begins.
- 2 The exercise-plus-ischemia test is repeated after pretreatment with the test drug
- 3 Finally, a second control (saline) exercise plus ischemia test is performed 1 week after the last drug test. At least 5 days are intermitted between drug treatments. Drugs are given in a random order.

Refractory Period Determination

On a subsequent day, the effective refractory period is determined using a Medtronic model 5325 programmable stimulator, both at rest and during myocardial ischemia. The heart is paced for 8 beats (S₁; in-

trastimulus interval, 300 ms; pulse duration, 1.8 ms at twice-diastolic threshold of ~ 6 mA). The intrastimulus interval is progressively shortened between the last paced beat and a single extrastimulus (S_2). The refractory period represents the shortest interval capable of generating a cardiac response and is measured using either the left or right ventricular electrodes. This procedure is completed within 30 s. Once the control values are determined, refractory period measurements are repeated after the standard drug glibenclamide (1.0 mg/kg i.v.), or the test drug. After the completion of these studies, refractory period is determined during myocardial ischemia (2-min occlusion of the left circumflex coronary artery) ~ 60 s after the onset of the coronary occlusion.

Reactive Hyperemia Studies

The K_{ATP} has been implicated in vascular regulation, particularly CBF (Aversano et al. 1991; Belloni and Hintze 1991; Daut et al. 1990). Therefore, the effects of standard and test drug on the response to brief interruptions in CBF are also evaluated. Animals are placed on a laboratory table, and the left circumflex coronary is occluded three or four times for 15 s. At least 2 min (or until CBF had returned to preocclusion base line) elapse between occlusions. The occlusions are then repeated 5 min after standard and test drug. On the subsequent day, the studies are repeated with the drug that had not been given the previous day.

EVALUATION

All hemodynamic data are recorded on a Gould model 2800S eight-channel recorder (Cleveland, OH) and a Teac model MR-30FM tape recorder (Tokyo, Japan). Coronary blood flow is measured with a University of Iowa Bioengineering flowmeter model 545 C-4 (Iowa City, IA). The rate of change of left ventricular pressure [$d(LVP)/dt$] is obtained by passing the left ventricular pressure through a Gould differentiator that has a frequency response linear to > 300 Hz. The data are averaged over the past 5 s of each exercise level. The coronary occlusion data are averaged over the last 5 s before and at the 60-s line point (or VF onset) after occlusion onset. The total area between the peak CBF and return to base line is measured for each 15-s occlusion, and the percent repayment is calculated. The reactive hyperemia response to each occlusion is then averaged to obtain one value for each animal. The data are then analyzed using analysis of variance for repeated measures. When the F ratio is found to exceed a critical value ($P < 0.05$), Scheffe's test is used to compare the mean values. The effects of

the drug intervention on arrhythmia formation are determined using a χ^2 test with Yates' correction for continuity. All data are reported as mean \pm SEM. Cardiac arrhythmias, PR interval and QT interval are evaluated at a paper speed of 100 mm/s. QT interval is corrected for HR using Bazett's method.

CRITICAL ASSESSMENT OF THE METHOD

Tests combining coronary constriction with physical exercise may resemble most closely the situation in coronary patients.

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A.5.0.9**Experimental Atrial Fibrillation****A.5.0.9.1****Atrial Fibrillation by Atrial Pacing in Dogs****PURPOSE AND RATIONALE**

Morillo et al. (1995) published a model of sustained atrial fibrillation by chronic rapid atrial pacing in dogs. Halothane-anesthetized mongrel dogs underwent insertion of a transvenous lead at the right atrial appendage that was continuously paced at 400 beats/min for 6 weeks. Two-dimensional echocardiography was performed to assess the effects of rapid atrial pacing on atrial size. Atrial vulnerability was defined as the ability to induce sustained repetitive atrial responses during programmed electrical stimulation. Effective refractory period (ERP) was measured at two endocardial sites of the right atrium. Sustained atrial fibrillation (AV) was defined as AF \geq 15 min. In animals with sustained AF, 10 quadripolar epicardial electrodes were surgically attached to the left and right atria. The local atrial fibrillatory cycle length (AFCL) was measured in a 20-s window. Marked biatrial enlargement was documented after 6 weeks of continuous rapid atrial pacing. An increase in atrial area of at least 40% was necessary to induce sustained AF.

More studies using this method were performed by Gaspo et al. (1997a, 1997b), Yue et al. (1997), Sun et al. (1998), and Nattel and Li (2000).

Shiroshita-Takeshita et al. (2004) studied the effect of drugs on atrial fibrillation promotion by atrial-tachycardia remodeling on dogs.

PROCEDURE

Mongrel dogs were anesthetized with ketamine (5.3 mg/kg i.v.), diazepam (0.25 mg/kg i.v.) and halothane (1.5%). Unipolar leads were inserted through jugular veins into the right ventricular apex and the right atrial appendage and connected to pacemakers (Medtronic) in subcutaneous pockets in the neck. A bipolar electrode was inserted into the right atrium for stimulation and recording during serial electrophysiological study. AV block was created by radiofrequency ablation of control ventricular response during atrial tachypacing. The right ventricular pacemaker was programmed to 80 beats/min.

After 24 h for recovery, a baseline closed-chest serial electrophysiological study was performed under ketamine/diazepam/isoflurane anesthesia, and then atrial tachypacing (400 beats/min) was initiated. The closed-chest electrophysiological study was repeated

at 2, 4, and 7 days of atrial tachypacing, and a final open-chest electrophysiological study was performed on day 8 under morphine-chloralose anesthesia.

Results of atrial tachypacing in drug-treated dogs were compared with results of dogs without treatment (controls).

Study Protocol

Dogs were anesthetized and ventilated mechanically. The atrial pacemaker was deactivated and a right atrium appendage effective refractory period was measured at basic lengths of 150, 200, 250, 300, and 360 ms with 10 basic stimuli (S_1) followed by a premature extra-stimulus (S_2) with 5-ms decrements. The longest S_1 - S_2 failing to capture defined the effective refractory period. AF was induced by atrial burst pacing at 10 Hz and 4 times threshold current. To estimate mean AF duration in each dog, AF was induced 10 times if AF duration was < 20 min and 5 times if AF lasted 20–30 min and then averaged. If AF lasted longer than 30 min, it was considered sustained and was terminated by DC cardioversion. A 20-min rest period was then allowed before continuing measurements.

For open-chest electrophysiological studies, dogs were anesthetized and ventilated mechanically. A femoral artery and both femoral veins were cannulated for pressure monitoring and drug administration. A median sternotomy was performed, and bipolar electrodes were hooked to the right atrial and left atrial appendages for recording and stimulation. A programmable stimulator (Digital Cardiovascular Instruments) was used to deliver twice-threshold currents. Five silicon sheets containing 240 bipolar electrodes were sutured onto the atrial surface (Fareh et al. 2001). Atrial effective refractory periods were measured as multiple basic cycle lengths in the right and left atrial appendages and at basic cycle length 300 ms in six additional sites: right and left atrium posterior wall, right and left atrium inferior wall, and right and left atrium Bachmann's bundle. Atrial fibrillation vulnerability was determined as the percentage of atrial sites at which AF could be induced by single extra-stimuli.

EVALUATION

Data are presented as mean \pm SEM. Multiple-group comparisons were obtained by ANOVA. AF duration data were analyzed after logarithmic transformation. Bonferroni-corrected *t* tests were used to evaluate individual-mean differences.

MODIFICATIONS OF THE METHOD

Verheule et al. (2004) described a canine model of atrial fibrillation due to chronic atrial dilatation.

Courtmanche et al. (1999) and Ramirez et al. (2000) published mathematical models of fibrillation-induced electrical remodeling and of canine atrial action potentials.

Pinto and Boyden (1999) reviewed electrical remodeling in ischemia and infarction.

Cabo and Boyden (2003) performed a computational analysis of electrical remodeling of the epicardial border zone in the canine infarcted heart.

Sakabe et al. (2004) reported that enalapril prevents perpetuation of atrial fibrillation by suppressing atrial fibrosis and over-expression of connexin43 in a canine model of atrial pacing-induced left ventricular dysfunction.

Baartscheer et al. (2005) induced combined volume and pressure overload in New Zealand white rabbits. In a first surgical procedure, volume overload was produced by rupture of the aortic valve until pulse pressure increased by about 100%, and after 3 weeks, pressure overload was created by suprarenal abdominal aortic constriction of 50%. In these animals, chronic inhibition of the Na⁺/H⁺ exchanger attenuated cardiac hypertrophy and prevented cellular remodeling in heart failure.

Using cultured atrial myocytes (HL-1 cells), Yang et al. (2005) found that rapid field stimulation (300 beats/min) causes electrical remodeling.

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A.5.0.9.2

Atrial Fibrillation in Chronically Instrumented Goats

PURPOSE AND RATIONALE

Atrial fibrillation is the most common tachyarrhythmia in humans. It causes palpitations, decreased cardiac output, heart failure and systemic thromboembolism, and is associated with significant mortality. Wijffels et al. (1995) demonstrated in a chronically instrumented conscious goat model, that episodes of atrial fibrillation may be self-perpetuating (“Atrial fibrillation begets atrial fibrillation”) and have suggested that there may be a purely electrophysiological explanation (termed atrial electrical remodeling) for the increase of atrial fibrillation with time. This model has been extensively used to study pathophysiological mechanism and the influence of drugs (Wijffels et al. 1997, 1999, 2000; Allesie et al. 1998; Tieleman et al. 1999; Duytschaever et al. 2000, 2005; Garratt and Fynn 2000; Van der Velden 2000a, 2000b; Veloso 2001; Brendel and Peukert 2003; Shan et al. 2004; Blaauw et al. 2004a).

Blaauw et al. (2004b) studied the efficacy and atrial selectivity of a blocker of the early ultrarapid compo-

ment of the delayed rectifier (I_{kur}) in remodeled atria of the goat.

PROCEDURE

Female goats weighing 52 ± 2 kg were used. According to the method of Duytschaever et al. (2001), Teflon-felt plaques with multiple electrodes were sutured onto the free wall of each atrium, Bachmann's bundle, and the left ventricle. All leads were tunneled subcutaneously to the neck and exteriorized by four 30-pole connectors. Experiments were started 3–4 weeks after surgery. Atrial fibrillation was induced by a fibrillation pacemaker (Wijffels et al. 1995).

The atria were paced with biphasic stimuli of 2 ms duration and $4 \times$ threshold. The atrial effective refractory period was measured at the free wall of the right and left atria during regular pacing (interval, 400–200 ms). Single interpolated stimuli were applied after eight basic stimuli, starting within the refractory period. The longest interval that failed to capture the atria (2-ms increments) was taken as the atrial effective refractory period. Atrial conduction velocity was measured along Bachmann's bundle during right atrial pacing. The distance over which conduction velocity was measured ranged from 3.5 to 5 cm.

The length of the fibrillation waves was determined at the right atrial free wall by measuring the refractory period and conduction velocity during AF. The refractory period was measured by slow, fixed-rate pacing (1 Hz), resulting in a series of single, randomly coupled, premature stimuli. Local capture of AF was evidenced by radial spread of activation from the pacing site and a short delay between stimulus and response. For each coupling interval, the percentage of capture was determined. The shortest interval capturing the atrium $\geq 50\%$ was taken as the refractory period. Conduction velocity was determined with a mapping electrode containing 5×6 electrodes (interelectrode distance, 4 mm) from the local conduction vectors within areas of 3×3 electrodes. At least 50 AF cycles were used to determine conduction velocity.

Inducibility of AF was measured at the right and left atria by single premature stimuli applied during regular pacing (400 ms). In case a premature beat induced a rapid irregular rhythm lasting > 1 s, AF was considered inducible. The AF cycle length was measured automatically by an algorithm detecting the negative intrinsic deflection of the fibrillation electrogram. A median value of 300 consecutive intervals was calculated. QT duration was measured during atrial pacing and persistent AF from either an epicardial electrogram or a precordial ECG. Because Bazett's for-

mula cannot be applied during AF, another approach was used to correct QT duration. In each goat, the relationship between the RR interval and QT duration was determined during 20 s of AF. The RR-QT relationship after drug administration was compared with the normal RR-QT relation.

The electrophysiological effects of the test drug and a conventional class III drug were measured before and after 48 h (1–4 days) of AF. The drugs were infused intravenously over 1 h, during which time AF cycle length was monitored. After 30 min of infusion, refractory period, conduction velocity, median RR interval, and QT duration were measured. Successful cardioversion was defined as termination of AF within ≤ 1 h of drug administration.

EVALUATION

Differences between groups were evaluated by paired Student's *t*-test or by two-way repeated ANOVA with post hoc Bonferroni's *t*-test. McNemar's test was used to compare AF inducibility. Changes in corrected QT duration were calculated by the one-sample *t*-test. Differences were considered significant at $P < 0.05$. Results are presented as mean \pm SEM.

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A.5.0.9.3

Influence on Ultrarapid Delayed Rectifier Potassium Current in Pigs

PURPOSE AND RATIONALE

Blockade of cardiac potassium channels and the resulting prolongation of repolarization and refractoriness is the mode of action of class III antiarrhythmic drugs. In the human heart, the ultrarapid delayed rectifier potassium current (I_{Kur}) was identified in atrial, but not in ventricular tissue. It appears to contribute to action potential repolarization (Wang et al. 1993; Li et al. 1996) in the atrium but not the ventricle. The molecular correlate of the human cardiac ultrarapid delayed rectifier potassium current seems to be the Kv1.5 protein (Fedida et al. 1993; Wang et al. 1993; Feng et al. 1997, 1998).

Since prolongation of ventricular repolarization seems to be invariably associated with proarrhythmia (early afterdepolarizations leading to torsades de pointes arrhythmia) as shown with available potassium channel blockers (class III drugs) such as the highly se-

lective and potent I_{Kr} -channel blocker dofetilide (Torp-Pedersen et al. 1999), blockade of a cardiac current that is exclusively present in the atria is highly desirable as it is expected to be devoid of ventricular proarrhythmic effects. Therefore, the atrial Kv1.5 channel is a highly attractive target in the search for new and safer atrial antiarrhythmic drugs (Nattel and Singh 1999).

In a series of studies, Wirth and Knobloch (2001), Knobloch et al. (2002, 2004), and Wirth et al. (2003) investigated electrophysiological and antiarrhythmic effects of I_{Kur} channel blockers on left versus right pig atrium *in vivo* in comparison with I_{Kr} blockers in pigs.

PROCEDURE

Surgery

Castrated male pigs (24–30 kg) of the German Landrace were premedicated with 3 ml Rompun 2% i.m. (xylazine HCL, 23.3 mg/ml = 3 mg/kg i.m.) and 6 ml Hostaket (ketamine HCL, 115 mg/ml = 20 mg/kg i.m.) and anesthetized with an i.v. bolus of 5 ml Narcoren (pentobarbital, 160 mg/ml = 25–30 mg/kg i.v.) followed by a continuous intravenous infusion of 12–17 mg/kg per h pentobarbital. Animals were ventilated with room air and oxygen by a respirator (ABV-Intensiv; Stephan, Gackebach, Germany). After a left thoracotomy the lung was retracted, the pericardium incised and the heart suspended in a pericardial cradle. Bipolar body surface ECG was recorded using subcutaneous needle electrodes in the classical lead II or lead III arrangement.

Atrial Effective Refractory Period Measurements

Atrial effective refractory period (ERP) measurements at different basic cycle lengths (BCL 240/300/400 ms) were performed (Wirth and Knobloch 2001). Atrial responses to the pacing procedure were visualized via monophasic action potential (MAP) from the left and right atrium as will be described below. A conditioning train of ten basic stimuli (S1) at twice-diastric pacing threshold was followed by a diastolic extrastimulus (S2, pulse duration 1 ms) starting about 30 ms above the expected ERP with a 5-ms decrement (UHS 20, universal heart stimulator; Biotronik, Berlin, Germany). The longest coupling interval unable to elicit a propagated atrial response was taken as the atrial ERP.

MAP Recording Sites and Atrial Pacing Electrodes

Left atrial ERP was measured via a MAP pacing catheter (EP Technologies, Model 1675; Boston Scientific, La Garenne-Colombes, France), which was fixed in each pig in the middle of the left atrial free wall in

an approximately perpendicular position by a holding device (Yuan et al. 1994). The tip of the MAP pacing catheter was covered by a sponge. Programmed stimulation was performed by the MAP pacing catheter. Right atrial MAP for ERP measurement was taken from the endocardium of the right atrium also via a steerable MAP pacing catheter. The catheter, inserted via the V. femoralis, was used for atrial stimulation too. Its position in the right atrium was checked by palpitation and by the typical atrial MAP morphology and duration and, additionally, by short rapid atrial pacing at a BCL of 240 ms, which the ventricle was not able to follow 1:1 as indicated by frequent *p*-waves dissociated from the QRS complex. There were no significant differences between endocardial and epicardial ERP measurements in the free walls of either atrium at baseline and after drug.

Left Atrial Vulnerability

During the ERP measurement procedure the mere S2-extrastimulus, which followed the ten conditioning S1 stimuli during the ERP-measurement procedure, frequently triggered runs of atrial tachycardia in the left, not the right, atrium. The occurrence of tachycardias was primarily unintended, but then exploited as a parameter for the judgement of the antiarrhythmic efficacy of compounds (referred to as left atrial vulnerability). Whether or not a run of S2-triggered atrial tachyarrhythmia occurred during the ERP-measurement procedure at a given BCL was noted. The occurrences of triggered tachyarrhythmias were summed up for the three BCLs tested over three time points (during a 30-min period before or after a drug). Thus, the maximal occurrence of S2-tachyarrhythmias during the control or drug period in an individual animal was nine.

Drugs

Drugs were dissolved in polyethyleneglycol (PEG) 400 (Riedel-de Haen, Seelze, Germany) and administered i.v. over 5 min in a volume of 3 ml. Vehicle was injected at least 30 min before each drug. For each drug a separate group of pigs was used.

EVALUATION

All data were presented as means \pm SEM. Two-way ANOVA for repeated measures followed by Student's *t*-test was used for the calculation of statistically significant differences between left and right atrial ERP prolongations at the three basic cycle lengths and the inhibition of left atrial vulnerability. A value of $P < 0.05$ was accepted as significant. The longest ERP at each pacing rate after drug administration was taken and

expressed as absolute or percent increase from vehicle control. Interatrial difference in refractoriness was calculated as the difference between the left and right atrial ERP.

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A.5.0.10

Characterization of Anti-Arrhythmic Activity in the Isolated Right Ventricular Guinea Pig Papillary Muscle

PURPOSE AND RATIONALE

According to Vaughan-Williams (1970) anti-arrhythmic drugs are divided into 4 different classes depending on their mode of action. Class I anti-arrhythmic

agents decrease the upstroke velocity of the action potential through blockade of Na⁺ channels. Class II drugs block β -receptors. Class III anti-arrhythmic agents prolong action potential duration, presumably through blockade of K⁺ channels. Class IV anti-arrhythmic agents inhibit the slow calcium influx during the plateau of the action potential through Ca²⁺ channel blockade. These electrophysiological actions also have functional manifestations, e. g., Na⁺ channel blockade decreases excitability, K⁺ channel blockade lengthens refractory period, and Ca²⁺ channel blockade decreases tension of cardiac muscle. A simple and accurate non-microelectrode method is necessary to identify and classify potential anti-arrhythmic drugs into the classes I, III, and IV. In right ventricular guinea pig papillary muscle developed tension (DT), excitability (EX), and effective refractory period (ERP) are measured.

PROCEDURE

Guinea pigs of either sex weighing 200–400 g are stunned, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed into a container of prewarmed, preoxygenated physiologic solution and the pericardium, atria, and other tissues are removed. The heart is then pinned to a dissection dish, and the right ventricle is opened. The tendinous end of the papillary muscle is ligated with a silk thread, and the chordae tendinae are freed from the ventricle. The opposite end of the papillary muscle is then cut free close to the ventricular wall. The non-ligated end of the papillary muscle is clamped into a tissue holder, the end of which is a leucite block containing platinum wire field electrodes.

The preparation is transferred to a tissue bath containing 75 ml of a physiological salt solution that is gassed continuously with 95% O₂/5% CO₂ and maintained at a temperature of 35°C and a pH of 7.4. The silk thread is used to connect the muscle to a Grass FT03C force transducer. An initial resting tension of 1 g is established. Muscles are field stimulated to contract isometrically. The stimulus duration is 1 ms, the frequency 1 Hz, and the voltage twice threshold. Pulses are delivered with the use of a Grass S88 constant voltage stimulator, and developed tension is recorded with the use of a polygraph recorder. The preparation is equilibrated in this manner for 90 min with bath solution changes every 15 min. Control measurements of the force-frequency curve, stimulus strength-duration curve and the effective refractory period are made following the 75 min bath exchange, i. e., during the last 15 min of equilibration.

The force-frequency curve is obtained by measuring developed tension over a range of stimulation frequencies (0.3, 0.5, 0.8, 1.0, and 1.2 Hz). The tissue is contracted for 90 s at each of these frequencies with a brief period of stimulation at 1.5 Hz inserted between increments. The purpose of the 1.5 Hz insert is to keep “pacing history” constant as well as to minimize progressive, nonspecific depression during the lower frequency stimulation series. Both pre- and postdrug developed tension (at each frequency) are expressed as a percentage of the predrug developed tension at 1 Hz. The percent change in post treatment (versus pretreatment) developed tension at 1 Hz is used to quantitate an agent’s inotropic effect.

The stimulus strength-duration curves are determined by varying the stimulus duration (0.1, 0.4, 0.8, 1.0, 1.5, 3.0, and 3.4 ms) and finding the threshold voltage that produced a 1:1 correspondence between stimulus and response at each duration. The degree of shift in the strength-duration curve is measured by computing the area between the pre- and post-treatment curves. The boundaries for the area are determined by the first (*x*-axis parallel) and the last (*x*-axis perpendicular) durations and by lines from the origin to the second and fourth durations.

Effective refractory period (ERP) is measured at 1 Hz using twin pulse stimuli. After every 8–10 pulses, a second delayed stimulus (*S*₂) identical to the basic drive pulse (*S*₁) is introduced. This procedure is repeated, shortening the delay (*S*₁–*S*₂) by 5 ms increments. The value of the ERP is taken as the longest delay (*S*₁–*S*₂) for which there is a single response to twin pulses. The change in ERP is computed as the difference (ms) between the pre- and post-treatment ERP values.

At the conclusion of the 90 min predrug equilibration period, an aliquot of the test drug designed to achieve the desired final concentration is added to the bath. The tissue must equilibrate for 1 h in the drug solution before postdrug measurements of the force-frequency curve, stimulus strength-duration curve, and effective refractory period are obtained.

EVALUATION

The changes in effective refractory period (ERP) (post treatment minus pretreatment), the degree of shift in the strength-duration curve (geometrical area between pre- and post-treatment curves), and the percent changes in post treatment developed tension at 1 Hz are calculated. The results of these calculations are used to classify the compound as a class I, III, or IV anti-arrhythmic agent on the basis of its effect on de-

veloped tension, excitability, and effective refractory period. An upward and right shift of the strength-duration curve (decrease in excitability) is characteristic for a class I anti-arrhythmic agent, such as disopyramide. Selective prolongation of effective refractory period is characteristic for class III anti-arrhythmic agents, such as sotalol. Depression of developed tension, and/or flattening or reversal of the force-frequency curve is characteristic for a class IV anti-arrhythmic agent, such as verapamil.

CRITICAL ASSESSMENT OF THE METHOD

The model of the electrically stimulated isolated guinea pig papillary muscle is a simple method to classify anti-arrhythmic agents. Some drugs have multiple actions and, therefore, belong in more than one class. For further characterization analysis of the action potential is necessary.

MODIFICATIONS OF THE METHOD

O'Donoghue and Platia (1991) recommended the use of monophasic action potential recordings for the evaluation of anti-arrhythmic drugs.

Shibuya et al. (1993) studied the effects of the local anesthetic bupivacaine on contraction and membrane potential in isolated canine right ventricular papillary muscles. From analysis of action potential it is concluded that at low concentrations contraction is depressed mainly due to a Na^+ channel block, whereas at high concentrations also Ca^{2+} channels may be blocked.

Kodama et al. (1992), Maryuama et al. (1995) studied the effects of potential antiarrhythmics on maximum upstroke velocity and duration of action potential in isolated right papillary muscles of guinea pigs as well as the influence of these agents on single ventricular myocytes.

Borchard et al. (1982) described a method for inducing arrhythmias or asystolia by the application of 50 Hz alternating current (ac) to electrically driven isolated left atria and right papillary muscles of the guinea pig. An increase in driving frequency from 1 to 3 Hz effected a significant reduction of the threshold of ac-arrhythmia in guinea pig papillary muscle, but no change in atria. A decrease in temperature from 31°C to 25°C and an increase in Ca^{2+} from 1.25 to 5 mmol/l elevated the threshold for ac-arrhythmia and -asystolia. Fast sodium channel inhibitors increased threshold of ac-arrhythmia in left atria and papillary muscles, whereas the slow channel inhibitor verapamil was ineffective in concentrations up to 6 $\mu\text{mol/l}$.

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A.5.0.11

Action Potential and Refractory Period in Isolated Left Ventricular Guinea Pig Papillary Muscle

PURPOSE AND RATIONALE

Intracellular action potential in the left ventricular guinea pig papillary muscle is recorded after electrical stimulation. The stimulation frequency is varied in order to determine the refractory period. Resting potential, upstroke velocity, duration of action potential, threshold, refractory period and contractile force can be measured *in vitro*. Compounds which affect the duration of the effective refractory period may have anti-arrhythmic or pro-arrhythmic effects. In addition, the inotropic effect (positive or negative) of the test compound is determined.

PROCEDURE

Guinea pigs of either sex (Marioth strain) weighing 250–300 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The

left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Ringer solution oxygenated with carbogen (95% O₂/5% CO₂) at a temperature of 36°C is used.

A standard micro electrode technique is applied to measure the action potential via a glass micro electrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at an interval of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold.

The intracellular action potential is amplified, differentiated for registration of upstroke velocity (dV/dt) (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

The effects on fast sodium channels as well as on calcium channels can be studied. The former requires measurement of the normal action potential and the latter the slow action potential obtained at 30 mM K⁺. To estimate the relative refractory periods, the second stimuli are set in decremental intervals until contraction ceases. Relative refractory period is defined as the minimum time interval of two stimuli at which each of the stimuli is answered by a contraction. The stimulation threshold is also measured.

After an equilibrium time of 30 min the test compound is added. After 15 and 30 min the following parameters are compared with the predrug values:

- Resting potential mV
- upstroke velocity V/s
- duration of action potential ms
- stimulation threshold V
- refractory period ms
- contraction force mg

The organ bath is flushed thoroughly between two consecutive applications of increasing test drug doses.

EVALUATION

Contractile force [mm] and relative refractory period [ms] are determined before and after drug administration. $ED_{25\text{ms}}$ - and $ED_{50\text{ms}}$ -values are determined. $ED_{25\text{ms}}$ or $ED_{50\text{ms}}$ is defined as the concentration of test drug in the organ bath at which the relative refractory period is reduced or prolonged by 25 ms or 50 ms.

Since many anti-arrhythmic agents possess additionally negative inotropic effects, changes in the force of contraction are also determined.

ED_{50} values are calculated from log-probit analyses. Scores are allotted relative to the efficacy of standard compounds (lidocaine, propranolol, quinidine).

The following changes are indicators for anti-arrhythmic activity:

- increase of stimulation threshold
- decrease of upstroke velocity
- prolongation of action potential
- increase of refractory period.

Upstroke velocity and duration of action potential are used for **classification purposes**.

MODIFICATIONS OF THE METHOD

Tande et al. (1990) studied the electromechanical effects of a class III anti-arrhythmic drug on guinea pig and rat papillary muscles and atria using conventional microelectrode technique.

Shirayama et al. (1991) studied with a similar technique the electrophysiological effects of sodium channel blockers in isolated guinea pig left atria.

Dawes (1946) described a method of examining substances acting on the refractory period of cardiac muscle using isolated rabbit auricles.

The same method was recommended as first step of a screening program for quinidine-like activity by Schallek (1956).

Wellens et al. (1971) studied the decrease of maximum driving frequency of isolated guinea pig auricles after antiarrhythmic drugs and beta-blockers.

Salako et al. (1976) recorded electropotentials along the conducting system after stimulation of the proximal part of the His bundle in rabbits.

Brown (1989), Wu et al. (1989), Gwilt et al. (1991a, b) measured *in vitro* transmembrane action potential in Purkinje fibers and endocardial ventricular muscles from dogs.

Voltage clamp techniques in isolated cardiac myocytes from guinea pigs have been used by Wettwer et al. (1991).

Nygren et al. (2004) described heterogeneity of action potential durations in isolated mouse left and right atria recorded using voltage-sensitive dye mapping.

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A.6 Methods to Induce Cardiac Hypertrophy and Insufficiency

Animal models of cardiac hypertrophy and insufficiency have been reviewed by Hasenfuss (1988), Muders and Elsner (2000), and Vanoli et al. (2004).

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A.6.0.1 Cardiac Hypertrophy and Insufficiency in Rats

A.6.0.1.1 Aortic Banding in Rats

PURPOSE AND RATIONALE

Blood flow restriction of the aorta in rats induces not only hypertension but also cardiac hypertrophy within several weeks. Angiotensin converting enzyme inhibitors, even at subantihypertensive doses, but not other antihypertensive drugs, inhibit cardiac hypertrophy (Linz et al. 1991; Schölkens et al. 1991; Gohlke et al. 1992; Linz 1992a; Linz et al. 1996; Bruckschlegel et al. 1995; Ogawa et al. 1998).

PROCEDURE

Male Sprague Dawley rats weighing 270–280 g are fasted 12 h before surgery. Anesthesia is induced by i.p. injection of 200 mg/kg hexobarbital. The abdomen is shaved, moistened with a disinfectant and opened by a cut parallel to the linea alba. The intestine is moistened with saline and placed in a plastic cover to prevent desiccation. The aorta is prepared free from connective tissue above the left renal artery and underlaid with a silk thread. Then, a cannula no. 1 (0.9 × 40 mm) is placed longitudinally to the aorta and both aorta and cannula are tied. The cannula is removed, leaving the aortic lumen determined by the diameter of the cannula. The intestine is placed back into the abdominal cavity with the application of 5.0 mg rolitetracycline (Reverin). In sham-operated controls no banding is performed. The skin is closed by clipping.

The animals are treated once daily over a period of 6 weeks with doses of the ACE-inhibitor or other antihypertensive drugs found previously effective to lower blood pressure in rats. At the end of the experiment blood pressure is measured under hexobarbital anesthesia (200 mg/kg i.p.) via indwelling catheters in the left carotid artery. Blood pressure measurement in conscious rats with the conventional tail-cuff method is not possible due to the large pressure difference across the ligature. Therefore, only one measurement at the end of the study is possible. The hearts are removed, rinsed in saline until free of blood and gently blotted to dryness. Total cardiac mass is determined by weighing on an electronic balance to the nearest 0.1 mg. The atria and all adjacent tissues are trimmed off and the weight of the left ventricle including the septum as well as the

remaining cardiac tissue representing the right ventricle are determined separately. Weights are calculated per 100 g body weight.

EVALUATION

Total cardiac mass, weight of left and right ventricle of treated rats are compared with operated controls and sham-operated controls.

MODIFICATIONS OF THE METHOD

Uetmasu et al. (1989) described a simple method for producing graded aortic insufficiencies in rats and subsequent development of cardiac hypertrophy. Selective perforation of the right cup of the aortic valve or in combination with that of the left valve cup was performed using a plastic rod inserted from the right common carotid artery. Hypertrophy of the heart, but no hypertension or cardiac insufficiency, was observed.

Similar methods were used by Yamazaki et al. (1989) to study the alterations of cardiac adrenoceptors and calcium channels subsequent to aortic insufficiency, by Umemura et al. (1992) to study baroreflex and β -adrenoceptor function and by Ishiye et al. (1995) to study the effects of an angiotensin II antagonist on the development of cardiac hypertrophy due to volume overload.

Hyperplastic growth response of vascular smooth muscle cells in the thoracic aorta was found following induction of acute hypertension in rats by aortic coarctation by Owens and Reidy (1985). Changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with aortic banding were studied by Feldman et al. (1993). Muders et al. (1995) produced aortic stenosis in rats by placing a silver clip (inner diameter 0.6 mm) on the ascending aorta. Schunkert et al. (1995) studied alteration of growth responses in established cardiac pressure overload hypertrophy in rats with aortic banding. **Prevention of cardiac hypertrophy after aortic banding** by ACE inhibitors probably mediated by bradykinin could be shown (Linz et al. 1989, 1992a, b, 1993, 1994; Linz and Schölkens 1992; Schölkens et al. 1991; Weinberg et al. 1994).

Weinberg et al. (1997) studied the effect of angiotensin AT₁ receptor inhibition on hypertrophic remodelling and ACE expression in rats with pressure-overload hypertrophy due to ascending aortic stenosis.

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A.6.0.1.2

Chronic Heart Failure in Rats

PURPOSE AND RATIONALE

Rat models of heart failure were reviewed by Muders and Elsner (2000). Chronic heart failure can be induced in rats by occlusion of coronary arteries. One of the first reports was by Selye et al. (1960). More recent reports are by Pfeffer et al. (1979), Hodsman et al. (1988), Van Veldhuisen et al. (1994, 1995), Kajstura et al. (1994), Gómez et al. (1997), Liu et al. (1997a, 1997b), and Jadavo et al. (2005).

Itter et al. (2004) described a model of chronic heart failure (CHF) in spontaneously hypertensive rats.

PROCEDURE

Study Design

Adult male 4-month-old SHR/NHsd and WKY/NHsd rats (Harlan Sprague Dawley, Winkelmann, Germany) weighing 250–300 g were used. Cardiovascular failure was induced by permanent (8 weeks) occlusion of the left coronary artery 2 mm distal to the origin from the aorta resulting in a large infarction of the free left ventricular wall.

Eight weeks after surgery, parameters indicating CHF were measured. Cardiac hypertrophy, function, and geometric properties were determined by the “working heart” mode and *in vivo* determinations by MRI and heart weight. Hydroxyproline/proline ratio was measured as an indicator of heart fibrosis.

Surgery

The rats were anesthetized with a mixture of ketamine/xylazine (35/2 mg/kg) i.p. The left ventrolateral thorax was shaved and prepared to create a disinfected surgical access area. When a stable anesthesia was achieved the animals were placed on a small animal operation table, intubated and ventilated with room air using a small animal ventilator (KTR-4, Hugo Sachs Elektronik, March-Hugstedten, Germany). The level of anesthesia was deemed as adequate following loss of the pedal withdrawal reflex and absence of the palpebral reflex. The tidal volume was adjusted at 3–5 ml and the ventilation rate was 40 breaths/min. Left thoracotomy was performed via the third intercostal space. The heart was exposed and the pericardium opened. The left main coronary artery was ligated with Perma-Hand silk 4–0 USP (Ethicon, Norderstedt, Germany) near its origin at the aorta (2 mm distal to the edge of the left atrium). Ligation resulted in infarction of the free left ventricular wall. Ligation was deemed successful when the anterior wall of the left ventricle turned pale. At this point the lungs were hyperinflated by increasing the positive end-expiratory pressure, and the chest was closed. The rats were placed on a heating pad. They were continuously monitored until they start moving in their cages. To avoid ventricular arrhythmias, lidocaine (2mg/kg i.m.) was given before surgery. The sham procedure consisted of opening the pericardium and placing a superficial suture in the epicardium of the LV. To prevent acute lung edema, the rats received furosemide 2 mg/kg twice daily for 3 days via the drinking water.

Measurements at the End of the Study

Before killing the animals 8 weeks after MI, non-invasive sequential nuclear magnetic resonance (NMR) measurements of heart geometric properties were done. Thereafter the animals were anesthetized with pentobarbitone (180 mg/kg i.p.) and subsequently heparinized (Heparin sodium 500 IU/100 g body weight i.p.). Once stable anesthesia was achieved (stage III 3, reflexes absent), the animals were connected to an artificial respirator via a PE tube inserted into the trachea and ventilated with room air. The right carotid artery was cannulated with a polyethylene catheter to monitor mean blood pressure, systolic blood pressure, diastolic blood pressure and heart rate over a stable time course of 10 min.

A transverse laparotomy and a right anterolateral thoracotomy were performed, and the heart was rapidly removed for the evaluation of its function in the working heart mode. Thereafter the heart weight, and

the left and right ventricular weights were determined. For infarct size determination the left ventricle was sectioned transversely into four slices from the apex to the base. The infarct size was determined by planimetry and expressed as a percentage of LV mass. Lung weight and further lung histology sections were evaluated. Hydroxyproline/proline ratio was determined in paraffin-embedded slices of the left ventricle.

Magnetic Resonance Imaging

The animals were monitored by MRI at day 7 and day 42 post-MI. The rats were anesthetized with a mixture of 1% halothane and 30/70 N₂O/oxygen with a specially manufactured rat mask. The fully anesthetized rats (phase III 3) were placed on a cradle made of Plexiglas in a supine position. Respiration and ECG were monitored continuously. MRI experiments were performed according to Rudin et al. (1991). The images were acquired by a spinecho sequence SE (500/20), the field of view was 50 mm, the image resolution was 256 × 256 pixels with a dimension of 0.2 × 0.2 mm. Four adjacent transverse slices were recorded; slice thickness was 1.5 mm. Before the acquisition of data, a coronary pilot scan was measured for adequate positioning of the transverse slices. MRI data acquisition was gated to the cardiac cycle by a Physiograd SM 785 MR monitoring system (Bruker, Karlsruhe, Germany). Two sets of transverse images were acquired, one at end-systole and another at end-diastole. End-diastole was defined as the image obtained 8 ms after the onset of the R wave of the ECG, corresponding to the largest cavity area. End-systole was defined as the image with the smallest LV cavity area. The image analysis was done using Bruker software (Karlsruhe, Germany). The parameters of left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), septum size, infarct size, ejection fraction (EF), left ventricular chamber diameter (r) and circumference were measured. EF was estimated in percentage terms by the subtraction LVEDV–LVESV. After the procedure the rats were ventilated with oxygen, the mask was replaced, and they were brought back into their cages. They were monitored until they started moving in the cage.

Blood Pressure/Heart Rate

The animals were anesthetized with pentobarbitone (180 mg/kg i.p.) and subsequently heparinized (Heparin sodium 500 IU/100 g body weight i.p.). Once stable anesthesia was achieved, the animals were connected to an artificial respirator via a PE tube inserted into the trachea and ventilated with room air. The

right carotid artery was cannulated with a polyethylene catheter. The catheter was connected to a PLUGSYS measuring system (Hugo Sachs Elektronik, March-Hugstedten, Germany) to monitor mean blood pressure, systolic blood pressure, diastolic blood pressure and heart rate over a stable time course of 10 min.

Working Heart

For the final investigations, the heart of the anesthetized rat was rapidly removed and immersed in physiological buffer chilled to 4°C. The aorta was dissected free and mounted onto a cannula (internal diameter: 1.4 mm) attached to a perfusion apparatus. The hearts were perfused according to the method of Langendorff with an oxygenated (95% O₂/5% CO₂) non-circulating Krebs–Henseleit solution of the following compositions (mM): NaCl, 118; KCl, 4.7; CaCl₂, 2.52; MgSO₄, 1.64; NaHCO₃, 24.88; KH₂PO₄, 1.18; glucose, 5.55; and Na-pyruvate, 2.0 at a perfusion pressure of 60 mmHg. Any connective tissue, thymus or lung was carefully removed. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for the determination of coronary flow and venous pO_2 measurements. The left atrium was cannulated via an incision of the left auricle. All pulmonary veins were ligated close to the surface of the atria. When a tight seal with no leaks had been established and after a 15-min equilibration period, the hearts were switched into the working mode, using a filling pressure (preload) of 12 mmHg in WKY/NHsd and 18 mmHg in SH rats. The afterload pressure was 60 mmHg in WKY/NHsd and 80 mmHg in SH rats. After validation of the basis parameters the afterload pressure was enhanced in a cumulative manner from an additional 20 mmHg to 140 mmHg. Thereafter the isovolumetric maxima were determined by enhancing the preload pressure in steps of 5 mmHg to 30 mmHg. Flow and pressure signals for computation were obtained from the PLUGSYS-measuring system. Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 s, using the software Aquire Plus V1.21f (PO-NE-MAH, Hugo Sachs Elektronik, March-Hugstedten, Germany).

Determination of Infarct Size

After the evaluation of the external heart work, the total heart weight, the left and right ventricular weights were determined. The left ventricle was then sectioned transversely into four slices from the apex to the base. Eight pictures were taken of each rat heart, two from each slice. Total infarct size was determined by planimetry of the projected and magnified slices.

The area of infarcted tissue as well as the intact myocardium of each slice were added together and averaged. The infarcted fraction of the left ventricle was calculated from these measurements and expressed as a percentage of the LV mass. The left ventricular perimeter, diameter, infarct scar length, as well as wall thickness and infarct wall thinning were determined as well. According to Pfeffer et al. (1985) and Pfeffer and Pfeffer (1987), rats with infarct sizes < 20% and > 40% were excluded from the study.

Lung Histological Determination

After lung weight determination, the organ was immersed in 4% formalin (pH 7.0–7.5; 0.1 M). The lung was cut into small pieces, dehydrated and embedded in paraffin. Hematoxylin and eosin (HE) sections were evaluated by light microscopy.

Hydroxyproline/Proline Ratio

After embedding, the rest of the fixed left ventricular tissue was freeze-dried. Proline and hydroxyproline was then analyzed according to the method of López de León and Rojkind (1985) and the ratio of both were calculated.

REFERENCES AND FURTHER READING

The data are given as mean \pm SEM. Statistics were performed using the SAS system statistics package (SAS Institute, Cary, N.C., USA) with a sequential rejection *t*-test.

MODIFICATIONS OF THE METHOD

Jain et al. (2000) studied the effects of angiotensin II receptor blockade after coronary ligation and exercise training on treadmill in rats.

Medvedev and Gorodetskaya (1993) induced heart failure in rats by microembolization of coronary vessels with 15- μ m plastic microspheres.

Katona et al. (2004) found that selective sensory denervation by capsaicin aggravates adriamycin-induced cardiomyopathy in rats.

A simple and rapid method of developing high output heart failure and cardiac hypertrophy in rats by producing **aorticaval shunts** was described by Garcia and Diebold (1990). Rats weighing 180–200 g were anesthetized with 30 mg/kg i.p. pentobarbitone. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two-thirds caudal to the renal artery and one-third cephalic to the aortic bifurcation with an 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating into

the vena cava. A bulldog vascular clamp was placed across the aorta caudal to the left renal artery. Once the aorta was clamped, the needle was fully withdrawn and a drop of cyanoacrylate glue was used to seal the aorta-punctured point. The clamp was removed 30 s later. The patency of the shunt was verified visually by swelling vena cava and the mixing of arterial and venous blood. The peritoneal cavity was closed with silk thread stitches and the skin with metallic clips. Rats with aorta-caval shunts developed cardiac hypertrophy with significantly higher absolute and relative heart weights.

Other studies with aorticaval shunts in rats were published by Flaim et al. (1979) and Liu et al. (1991).

Isoyama et al. (1988) studied myocardial hypertrophy after creating aortic insufficiency in rats.

Terlink et al. (1998) studied ventricular dysfunction in rats with diffuse isoproterenol-induced myocardial necrosis.

Sabbah et al. (1991) described a model of chronic heart failure produced by multiple sequential coronary microembolizations in **dogs**.

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A.6.0.2

Cardiac Hypertrophy and Insufficiency in Mice

A.6.0.2.1

Cardiac Hypertrophy in Mice

PURPOSE AND RATIONALE

Rockman et al. (1991, 1993) developed a model of ventricular hypertrophy in the intact mouse by use of microsurgical techniques.

PROCEDURE

Eight-week-old adult mice weighing 18–22 g are anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine, 5 mg/kg xylazine, and 2.5 mg/kg morphine. Animals are placed under a dissecting microscope in the supine position and a midline cervical incision is made to expose the trachea and carotid arteries. After endotracheal intubation, the cannula is connected to a volume cycled rodent ventilator on supplemental oxygen with a tidal volume of 0.2 ml and a respiratory rate of 110 per min. Both left and right carotid arteries are cannulated with flame stretched PE50 tubing. Catheters are connected to modified P50 Statham transducers.

The chest cavity is entered in the second intercostal space at the left upper sternal border through a small incision and the thymus is gently deflected out of the field of view to expose the aortic arch. After the transverse aorta is isolated between the carotid arteries, it is constricted by a 7.0 nylon suture ligature against a 27-gauge needle, the latter being promptly removed to yield a constriction of 0.4 mm diameter and provide a reproducible transverse aortic constriction of 65–75%.

The hemodynamic effects of acute and chronic constriction are followed by monitoring the pressure gradient between the two carotid arteries in anesthetized animals. Systolic and mean arterial pressure at baseline, during total occlusion when the ligature is tied, and early (15 min) and late (7 days) after transverse aortic constriction are recorded. The increase in systolic pressure provides an adequate mechanical stimulus for the development of cardiac hypertrophy.

To confirm myocardial hypertrophy, both sham-operated and aortic-constricted hearts are examined 7 days after operation. Hearts examined for *cell size* are perfused with 4% paraformaldehyde/1% glutaraldehyde through the apex, immersed in osmium tetroxide, dehydrated in graded alcohols, and embedded in araldite. Tissue blocks are sectioned at a thickness of 1 μ m, mounted on slides, and stained with tolu-

idine blue. Cell areas are measured by manually tracing the cell outline on an imaging system connected to a computer.

At the end of the experiment, mice were sacrificed in anesthesia, heart excised and weighed, the atria and ventricles separately frozen in liquid nitrogen for Northern blot analysis. Total RNA is extracted by a single step extraction with guanidinium thiocyanate. The RNA is size fractionated by agarose gel electrophoresis, transferred to nylon membranes by vacuum blotting, and hybridized with the appropriate complementary DNA probes labeled with ^{32}P by random priming to a specific activity of $0.95\text{--}1.2 \times 10^6$ cpm/ng.

EVALUATION

Variables measured are expressed as mean \pm SD. Statistical significance of differences between sham-operated and thoracic aortic-constricted animals is assessed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Dom et al. (1994) studied myosin heavy chain regulation and myocytes' contractile depression after LV hypertrophy in aortic-banded mice.

Okada et al. (2004) subjected mice to transverse aortic constriction. Echocardiographic analysis demonstrated cardiac hypertrophy and failure 1 and 4 weeks after surgery. Cardiac expression of endoplasmatic reticulum chaperones was significantly increased indicating that pressure overload by transverse aortic constriction induced prolonged endoplasmatic reticulum stress.

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A.6.0.2.2

Chronic Heart Failure in Mice

PURPOSE AND RATIONALE

Several authors reported the development of murine models of cardiac failure (Kaplan et al. 1994; Rockman et al. 1994; Balasubramaniam et al. 2004; Suzuki et al. 2004; Walther et al. 2004; Wang et al. 2004; Liao et al. 2005).

Xu et al. (2004) studied cardioprotection in mice with heart failure by dual inhibition of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP).

PROCEDURE

Mice with a targeted deletion of the B₂ kinin receptor gene or C57BL/6J mice at an age of 10–12 weeks were anesthetized with 50 mg/kg sodium pentobarbital i.p., intubated and ventilated with room air using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space; the lungs were retracted to expose the heart, and the pericardium was opened. The left anterior descending coronary artery was ligated with an 8–0 nylon suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was considered successful when the anterior wall of the left ventricle turned pale and an obvious ST segment elevation was observed. The lungs were inflated by increasing positive end-expiratory pressure and the thoracotomy site was closed. Sham-operated mice were subjected to the same procedure except that the suture around the left anterior coronary artery was not tied.

Systolic blood pressure was measured in conscious mice using a non-invasive computerized tail-cuff system. Cardiac geometry and function were evaluated with a Doppler echocardiographic system. LV diastolic dimension was measured and ejection fraction was calculated from

$[(\text{LVAd} - \text{LVAs})/\text{LVAd}] \times 100$, where LVAd is LV diastolic area and LVAs is LV systolic area.

Four weeks after surgery, each strain was separated into one group treated with an ACE inhibitor, one group treated with a NEP inhibitor, one group treated with both inhibitors and one control group. All drugs were administered in drinking water for 20 weeks.

At the end of the study, all mice were anesthetized with pentobarbital and the heart stopped at diastole

by intraventricular injection of 15% KCl. The heart, lungs, and liver were weighed to assess hypertrophy and congestion. Infarct size was determined by Gomori trichrome staining and expressed as the ratio of the infarcted portion to total LV circumference.

Sections (6 μ m) from each slice were double-stained with fluorescein-labeled peanut agglutinin to delineate the myocyte cross-sectional area and interstitial space and rhodamine-labeled *Griffonia simplicifolia* lectin I to show the capillaries. To calculate interstitial collagen fraction, the total surface area (microscopic field), interstitial space (collagen plus capillaries), and area occupied by capillaries alone were measured by computer-assisted videodensometry.

After 20 weeks of treatment, plasma renin was measured.

EVALUATION

Data were expressed as mean \pm SE. Mortality rates were compared using χ^2 tests. For the echo, blood pressure, heart weight, lung weight, infarct size, plasma renin concentration, and histology data, paired or two-sample tests using non-parametric methods were used to perform all comparisons of interest.

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A.6.0.2.3

Transgenic Mice and Heart Failure

PURPOSE AND RATIONALE

Several hundreds of papers on transgenic mice and heart failure are published. Only a few can be mentioned here.

Chien (1995) described cardiac muscle diseases in genetically engineered mice.

Edwards et al. (1996) described severe cardiomyopathy in transgenic mice overexpressing the skeletal muscle myogenic regulator *myf5*.

Arber et al. (1997) found that MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure.

Graham et al. (1997) described a mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoforms of the adenine nucleotide translocator.

Iwase et al. (1997) studied cardiomyopathy in transgenic mice induced by overexpression of the cardiac stimulatory G protein α subunit.

Knollmann et al. (2000) reported remodeling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin.

Beggah et al. (2002) described reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes.

Verheule et al. (2004) found increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF- β 1.

Duncan et al. (2005) found that chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy.

Hartil and Charron (2005) reviewed mouse models where transgenic technology has been utilized to alter expression of genes involved in cardiac uptake and metabolism of either lipid or carbohydrate.

Hilfiker-Kleiner et al. (2005) reported that STAT3-knockout mice harboring a cardiomyocyte-restricted deletion of STAT3 showed enhanced susceptibility to cardiac injury caused by myocardial ischemia, systemic inflammation, or drug toxicity.

Sanbe et al. (2005) studied reversal of amyloid-induced heart disease in desmin-related cardiomyopathy.

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A.6.0.3

Cardiac Insufficiency in Guinea Pigs

PURPOSE AND RATIONALE

Congestive heart failure in man is characterized by cardiac hypertrophy, peripheral edema, lung and liver congestion, dyspnea, hydrothorax and ascites. Effective treatment is achieved by cardiac glycosides. Based on techniques reported by Selye et al. (1960) a method was developed to induce congestive heart failure in guinea pigs with symptoms very close to human pathology (Vogel and Marx 1964; Vogel et al. 1965).

PROCEDURE

Male guinea pigs weighing 250–400 g are used. The fur at the ventral thorax is shaved and the skin disin-

fectected. The animal is anesthetized with ether. The skin is cut with scissors on the left side at a length of 4 cm. The left musculus pectoralis is cut at the costal insertion and elevated. The fourth intercostal space is opened with two blunted forceps. The heart is pressed against the opening with the left hand. The pericardium is opened with a fine forceps and pulled back to the basis of the heart. The beating heart is extruded from the thorax wound by pressure with the left hand on the right thorax wall. A ring-shaped clamp covered with a thin rubber tube is placed around the basis of the heart keeping the heart outside of the thorax without closing off the blood circulation. A thread soaked with diluted disinfectant solution is placed as a loop around the apex of the heart and tightened so that the apical third of both ventricles is tied off. The degree of tightening of the loop is essential. Complete interruption of blood supply to the apical third resulting in necrosis has to be avoided as well as the loop's slipping off. Technical skill is necessary to place the loop around the beating heart into the correct position. After removal of the clamp the heart is placed back, the incision between the fourth and fifth costal rib closed and the musculus pectoralis placed over the wound. Intrathoracic air forming a pneumothorax is removed by pressure on both sides of the thorax. After application of an antibiotic emulsion the skin wound is closed. The surgical procedure has to be finished within a short period of time.

The animals develop symptoms of severe congestive heart failure with a death rate of 80% within 14 days. Lung weight and relative heart weight are significantly increased. Exudate in the thorax cavity and ascites amount between 3.5 and 7.5 ml with extreme values of 17.5 ml. Lung edema and liver congestion are found histologically. Peripheral edema and preterminal dyspnea and tachypnea are observed. When treated with various doses (0.1 to 100 μ g/kg) of cardiac glycosides s.c. or i.m. over a period of 14 days the symptoms of cardiac insufficiency, e. g., volumes of transudate as well as death rate, are dose-dependent diminished.

EVALUATION

From survival rate, ED_{50} values of cardiac glycosides can be calculated which are in the same dosage range as therapeutic doses in man.

CRITICAL ASSESSMENT OF THE METHOD

The experimental model in guinea pigs reflects very closely the symptoms of cardiac insufficiency in man, e. g., lung congestion, hydrothorax, liver congestion, ascites, peripheral edema and cardiac hypertrophy. The

therapeutic potency of cardiac glycosides can be evaluated with this method. Additional factors being known to enhance the symptoms of congestive heart failure in man, like salt load and diphtheria toxin, further increase mortality and hydropic symptoms. The method can be used for special purposes, however, it needs considerable training and technical skill.

MODIFICATIONS OF THE METHOD

Siri et al. (1989, 1991) produced left ventricular hypertrophy in the guinea pig by gradually increasing ventricular afterload. A mildly constricting band was placed around the ascending aorta of very young guinea pigs (225–275 g). With growth to 500–1000 g, left ventricular systolic pressure increased and ventricular hypertrophy developed. Only some of the animals developed dyspnea and severe ventricular dysfunction.

Kiss et al. (1995) studied the effects on Ca^{2+} transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure in guinea pigs. The descending aorta was banded for 4 and 8 weeks in adult guinea pigs.

Tweedle et al. (1995) assessed subrenal banding of the abdominal aorta as a method of inducing cardiac hypertrophy in the guinea pig.

Pfeffer et al. (1987) induced myocardial infarction in **rats** by ligation of the left coronary artery and found hemodynamic benefits and prolonged survival with long-term captopril therapy.

Acute ischemic left ventricular failure can be induced in anesthetized **dogs** by repeated injections of plastic microspheres into the left coronary artery (see A.3.2.4).

Huang et al. (1997) created congestive heart failure in **sheep** by selective sequential intracoronary injection of 90 μm microspheres under 1.5% isoflurane injection.

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A.6.0.4

Cardiomyopathic Syrian Hamster

PURPOSE AND RATIONALE

Cardiomyopathy in Syrian hamsters has been described by Bajusz et al. (1966), Bajusz and Lossnitzer (1968), Bajusz (1969), Bajusz et al. (1969a, b), Homburger and Bajusz (1970), Gertz (1972). The disease originates from an autosomal, recessively transmissible disorder, which leads to degenerative lesions in all striated muscles and in particular in the myocardium. Histopathological changes consist of myocytolytic necrosis followed by fibrosis and calcification. The evolution of the cardiomyopathic disease can be characterized by five distinct phases: A pre-necrotic stage, in which no pathology is evident, a time of active myocytolysis and cellular necrosis, a phase of fibrosis and calcium deposition, an overlapping period of reactive hypertrophy of the remaining viable myocytes, and a final stage of depressed myocardial performance and failure.

PROCEDURE

The model of cardiomyopathy in Syrian hamsters has been used by several authors. One has to note, that several strains of cardiomyopathic hamsters have been used: strain Bio 53:58 by Capasso et al. (1989, 1990) and by Chemla et al. (1992, 1993), strain BIO 14.6 by Tapp et al. (1989) and by Sen et al. (1990), strain CHF 146 CM by van Meel et al. (1989) and by Haleen et al. (1991), strain BIO82.62 by ver Donck et al. (1991), strain J-2-N by Kato et al. (1992), strain CHF 147 by Desjardins et al. (1989), Hanton et al. (1993).

Various experimental protocols have been described. Most authors use survival rate and heart weight as end point (e. g., van Meel et al. 1989; ver Donck et al. 1991; Hanton et al. 1993). Generally, the experiments are started with animals at an age of 120 to 200 days.

Capasso et al. (1989, 1990) studied the mechanical and electrical properties of cardiomyopathic hearts of Syrian hamsters using isolated left ventricular posterior papillary muscles.

Tapp et al. (1989) tested stress-induced mortality in cardiomyopathic hamsters by five consecutive daily 2-h periods supine immobilizations at 4°C.

Sen et al. (1990) tested the inotropic and calcium kinetic effects of calcium channel agonists and antagonists in primary cultures of isolated cardiac myocytes.

Haleen et al. (1991) tested the effects of an angiotensin converting enzyme inhibitor not only on survival, but also on left ventricular failure in the isolated Langendorff heart by measurement of left ventricular end-diastolic pressure, dP/dt_{max} and mean coronary flow.

Dixon et al. (1997) tested the effect of an AT₁ receptor antagonist on cardiac collagen remodelling in the cardiomyopathic Syrian hamster.

In addition to the effects on left ventricular papillary muscles strips, Chemla et al. (1992) tested the effects on diaphragm contractility in the cardiomyopathic Syrian hamster.

Whitmer et al. (1988) and Kuo et al. (1992) tested sarcolemmal and sarcoplasmic reticulum calcium transport in the cardiomyopathic Syrian hamster.

Nigro et al. (1997) identified the Syrian hamster cardiomyopathy gene.

Tanguay et al. (1997) tested the coronary and cardiac sensitivity to a vasoselective benzothiazepine-like calcium antagonist in isolated, perfused failing hearts of Syrian hamsters.

Bilate et al. (2003) recommended the Syrian hamster as a model for the dilated cardiomyopathy of Chagas' disease. Female hamsters were infected via the intraperitoneal route with *Trypanosoma cruzi* Y strain blood trypomastigotes. Survival was monitored, echocardiography was performed after 4 and 12 months, and histopathological examinations were carried out at the end of the study period.

CRITICAL ASSESSMENT OF THE METHOD

Positive effects of various drugs have been found in the cardiomyopathic hamster, such as cardiac glycosides, inotropic compounds, beta-blockers, calcium antago-

nists, and ACE-inhibitors. The specificity of the effects has to be challenged.

MODIFICATIONS OF THE METHOD

The **tight-skin (TSK) mouse** is a genetic model of pulmonary emphysema connected with right ventricular hypertrophy (Martorana et al. 1990; Gardi et al. 1994).

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A.6.0.5

Cardiac Failure in Rabbits

PURPOSE AND RATIONALE

Rabbit models of heart failure were reviewed by Mudders and Elsner (2000).

Rapid pacing was used by Masaki et al. (1994), Porsa et al. (1994), Eble et al. (1998), Li et al. (2003), and Rose et al. (2005); coronary artery ligation by Penock et al. (1997), Currie and Smith (1999), Romanic et al. (2001), and Miller et al. (2004); combined pressure and volume overload by Ezzaher et al. (1991), Mohammadi et al. (1997), Dekker et al. (1998), and Baartscheer et al. (2003a, 2003b); aortic insufficiency and aortic constriction by Bouanani et al. (1991) and Pogwizd et al. (1999); regurgitation after damage of the mitral valve by Gunawardena et al. (1999); regurgitation after aortic valve destruction by Magid et al. (1988, 1994), Yoshikawa et al. (1993), King et al. (1997), and Liu et al. (1998); Luchner et al. (2001) used a rabbit model of progressive left ventricular dysfunction to investigate differential expression of cardiac atrial natriuretic peptide and brain natriuretic peptide. Ventricular pacing-induced heart failure could be induced with a transvenously implanted pacemaker system.

PROCEDURE

Male rabbits (chinchilla bastard) underwent implantation of a programmable cardiac pacemaker (Medtronic Minix 8340, Minneapolis, Mn., USA). Under anesthesia (ketamine 60 mg/kg xylazine 5 mg/kg i.m.), the right internal jugular vein was dissected and cannulated with a single-lumen central venous catheter (Braun, Germany). The catheter was then advanced

into the right ventricle under pressure guidance. A transvenous screw-in pacemaker lead (Medtronic) was advanced through the catheter into the ventricular apex and implanted endocardially. The pacemaker was implanted subcutaneously into the right abdominal wall and the pacemaker lead was connected subcutaneously with the pacemaker. Rapid ventricular pacing-induced heart failure could be induced with a transvenously implanted pacemaker system. All rabbits were allowed to recover for at least 10 days after surgery before the pacemaker was started for the induction of heart failure. Proper pacemaker function was checked intraoperatively, at the time of programming, and subsequently all 10 days.

Rabbits (CHF group) underwent pacing with a step-wise increase of stimulation frequencies over 30 days. During the first 10 days, animals were paced at 330 beats/min (bpm). This protocol results in ELVD, as defined by significant LV systolic dysfunction with cardiac enlargement and decreased perfusion pressure but no clinical signs failure. The pacing rate was then increased to 360 bpm for 10 days and 380 bpm for another 10 days and ELVD evolved to CHF with further cardiac enlargement and further decreased perfusion pressure together with clinical signs of fluid retention (ascites). At baseline (control), after being paced at 330 bpm for 10 days (ELVD) and at the end of the protocol (CHF), conscious arterial pressure was measured invasively via the medial ear artery and a 2-D guided M-mode echocardiogram was obtained. At the end of the pacing protocol, rabbits were killed by i.v. euthanasia and tissue was rapidly harvested. Hearts were trimmed on ice, snap frozen in liquid nitrogen and stored at -80°C until further processing.

Echocardiography

A long and short-axis echocardiogram (HP Sonos 5500, 12 MHz probe) was performed under light sedation (5 mg midazolam i.m.) in a supine position from the left parasternal window. LV end-diastolic (LVEDd) and end-systolic (LVESd) dimensions and diastolic and systolic thickness of the left ventricular anterior wall (AEDth and AESTh) and posterior wall (PEDth and PESTh) as well as left atrial diameter (LAd) were determined from three repeated 2-D guided M-mode tracings using the ASE convention. From those measurements, fractional shortening (FS) was calculated as: $\text{FS} = (\text{LVEDd} - \text{LVESd}) / \text{LVEDd}$.

Analytical methods

For analysis of cardiac natriuretic peptide expression, mRNA was extracted from all atrial and left ventricular samples utilizing a commercial kit (Fastrack, Invitrogen).

As a probe for brain natriuretic peptide (BNP), a 750-bp *EcoRI/HindIII* DNA restriction fragment containing the gene for rabbit BNP was used.

EVALUATION

Results of the quantitative studies were expressed as mean \pm SEM. Comparisons between the control, ELVD and CHF groups were performed by analysis of variance (ANOVA) followed by Fisher's least significant difference test. Comparison between the atrial and LV tissues as well as between atrial natriuretic peptide (ANP) and BNP were performed by paired Student's *t*-test. Statistical significance was defined as $P < 0.05$.

MODIFICATIONS OF THE METHOD

Arnolda et al. (1985) studied adriamycin cardiomyopathy in the rabbit.

Klimtova et al. (2002) performed a comparative study of chronic toxic effects of daunorubicin and doxorubicin in rabbits.

Alexander et al. (1993) studied electrographic changes following corona-virus-induced myocarditis and dilated cardiomyopathy in rabbits.

Sanbe et al. (2005) described a transgenic model for human troponin I-based hypertrophic cardiomyopathy in the rabbit.

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A.6.0.6

Congestive Heart Failure in Dogs

PURPOSE AND RATIONALE

Several methods are described, to induce congestive heart failure in dogs, such as rapid ventricular pacing (Armstrong et al. 1986; Freeman et al. 1987; Wilson et al. 1987; Komamura et al. 1992, 1993; Perreault et al. 1992; Travill et al. 1992; Cheng et al. 1993; Redfield et al. 1993; Cory et al. 1994; Kiuchi et al. 1994; Ohno et al. 1994; Vatner et al. 1994; Wang et al. 1994; Williams et al. 1994; Eaton et al. 1995; Spinale et al. 1995; Wolff et al. 1995; Zile et al. 1995; Ravens et al. 1996; Shinbane et al. 1997; O'Rourke et al. 1999; Winslow et al. 1999).

Luchner et al. (1996) assessed circulating, renal, cardiac, and vascular angiotensin II in a canine model of rapid ventricular pacing-induced heart failure that evolves from early left ventricular dysfunction to overt congestive heart failure.

PROCEDURE

Male mongrel dogs underwent implantation of a programmable cardiac pacemaker (Medtronic). Under pentobarbital sodium anesthesia and artificial respiration, the heart was exposed via a small left lateral thoracotomy and pericardiotomy, and a screw-in epicardial pacemaker lead was implanted into the right ventricle. The pacemaker was implanted subcutaneously into the left chest wall and connected to the pacemaker lead. The dogs were allowed to recover for at least 10 days after surgery before the pacemaker was started. During the first 10 days dogs were paced at 180 beats/min (bpm), resulting in early left ventricular dysfunction as defined by significant systolic dysfunction with decreased cardiac output, cardiac enlargement, and increased filling pressures but maintained systemic perfusion pressure and renal sodium

excretion and no clinical signs of heart failure. The pacing rate was then increased weekly to 200, 210, 220 and 240 bpm, and early left ventricular dysfunction evolved to overt congestive heart failure with avid sodium retention and clinical signs of congestion. At baseline (control), after dogs had been paced at 180 bpm for 10 days and at the end of the protocol (overt CHF), urine was collected for measurement of sodium excretion; conscious mean arterial pressure was measured via a port catheter; a 2-D guided M-mode echogram was obtained; and arterial blood was drawn. Cardiac filling pressures and cardiac output were measured by the thermodilution method at baseline and at the end of the protocol. Arterial blood was collected in EDTA tubes for measurement of ANP, BNP, cGMP, PRA, aldosterone, and Ang II. After euthanasia, hearts were rapidly trimmed and left ventricles weighted for calculation of the index LV weight to body weight.

EVALUATION

Results were expressed as mean \pm SE. Comparison between the control, early LV dysfunction, and overt CHF were performed by ANOVA followed by Fisher's least significant difference test.

MODIFICATIONS OF THE METHOD

Kleaveland et al. (1988) and Nagatsu et al. (1994) used the technique of experimental **mitral regurgitation** in dogs to induce left ventricular dysfunction. A 30-cm, 7F sheath was introduced across the aortic valve through the carotid artery. A urologic calculus retrieval forceps was advanced through the sheath to the mitral valve apparatus and was used to sever chordae tendineae. When pulmonary capillary wedge pressure rose to 20 mmHg and forward stroke volume was reduced to 50% of its baseline, a ventriculogram was performed to confirm angiographically that severe mitral regurgitation had been created.

Dell'Italia et al. (1995) and Su et al. (1999) induced mitral regurgitation by percutaneous chordal rupture in dogs.

Kinney et al. (1991) published a method to induce acute, reversible tricuspid insufficiency in anesthetized dogs. A wire spiral is advanced through the atrioventricular canal from the right atrium. The spiral causes regurgitation by preventing complete apposition of the valve leaflets while permitting retrograde flow to occur through the spiral lumen. The degree of regurgitation can be controlled by the use of spirals of different size. Creation of tricuspid insufficiency was demonstrated

by onset of right atrial pressure V waves, a ballooning of the right atrium during ventricular systole, palpation of an atrial thrill, or by color Doppler echocardiography. The model is reversible and allows repeated trials of various grades of regurgitation.

Carlyle and Cohn (1983) described a non-surgical model of chronic left ventricular dysfunction. The method is accomplished by repetitive DC shock with a guidewire introduced percutaneously and positioned in the left ventricle along the intraventricular septum and an external paddle at the left ventricular apex.

McDonald et al. (1992) produced localized left ventricular necrosis without obstruction of the coronary blood flow in dogs by transmural direct-current shock.

Sabbah et al. (1991, 1993, 1994) and Gengo et al. (1992) produced chronic heart failure in dogs by multiple sequential intracoronary **embolizations with microspheres**. The dogs underwent three to nine intracoronary embolizations with polystyrene latex microspheres (70–102 μ m in diameter) performed 1–3 weeks apart. Embolizations were discontinued when left ventricular ejection fraction was less than 35%.

Vanoli et al. (2004) used multiple coronary microembolizations in dogs, whereby three to nine embolizations were performed 1 week apart. The first three embolizations consisted of 2 ml of microsphere suspension injected subselectively into either the left anterior descending or left circumflex coronary artery in an alternating fashion. Subsequent embolizations consisted of 3–6 ml of microspheres divided equally between the left anterior descending or left circumflex coronary artery until LV ejection fraction was <35%.

Magovern et al. (1992) described a canine model of left ventricular dysfunction caused by five weekly intracoronary infusions of **adriamycin**.

Koide (1997) described premonitory determinants of left ventricular dysfunction in a model of gradually induced pressure overload in dogs. Mongrel dogs were studied through 8 weeks of gradually imposed ascending aortic constriction with the use of a **novel banding technique**. During banding, an initial gradient of 30 mmHg was created. Before banding, at 2, 4, and 6 weeks after banding, hemodynamics and left ventricular mechanics were examined at cardiac catheterization; then the pressure overload was increased by tightening the band.

Valentine et al. (1988) and Devaux et al. (1993) described **X-linked muscular dystrophy in dogs** with cardiac insufficiency similar to Duchenne muscular dystrophy in men and recommended this as an animal model for cardiac insufficiency.

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Cardiac Failure in Pigs

PURPOSE AND RATIONALE

Cardiac failure was studied in pigs using several experimental procedures.

Chow et al. (1990) recommended rapid ventricular pacing in pigs as an experimental model of congestive heart failure.

Farrar et al. (1993) studied pacing-induced dilated cardiomyopathy in pigs. Congestive heart failure was produced by rapid ventricular pacing at 230 bpm for 1 week.

Spinale et al. (1990a, 1990b, 1991, 1992) examined the consequences of chronic supraventricular tachycardia on various parameters of ventricular dysfunction and subendocardial changes in pigs.

Carroll et al. (1995) investigated gene expression in a swine model of ventricular hypertrophy during pressure overload.

Multani et al. (2001) studied long-term angiotensin converting enzyme and angiotensin I-receptor inhibition in pacing-induced heart failure in pigs. Heart failure was induced by rapid atrial pacing (240 bpm for 3 weeks).

Kassab et al. (1993, 2000) investigated remodeling of right ventricular branches after hypertrophy in pigs.

Krombach et al. (1999) studied the effects of amlodipine in congestive heart failure in pigs at rest and after treadmill exercise.

PROCEDURE

Left thoracotomy was performed in Yorkshire pigs in anesthesia. Catheters connected to a vascular access port were placed in the thoracic aorta, the pulmonary artery, and the left atrium. The access ports were then placed in a subcutaneous pocket. A 20-mm flow probe was placed around the pulmonary artery immediately distal to the pulmonary catheter and the electrical connection exteriorized through the thoracolumbar fascia. A shielded stimulating electrode was sutured onto the left atrium, connected to a programmable pacemaker and buried in a subcutaneous pocket. The thoracotomy was closed in layers and the pleural space evacuated of air. After a 14- to 21-day recovery, measurements were performed under normal resting conditions and after exercise. The pacemakers were activated to 240 bpm for a period of 21 days. During the last 3 days, one group was treated with drug, the other served as control. At the day of the study, electrocardiograms were performed, and the pacemakers deactivated. After a 30-min stabilization period, 2-D and M-mode echocardiographic studies were used to image the left ventricle from the parasternal approach. Left ventricular fractional shortening was calculated as (end-diastolic dimension – end-systolic dimension)/diastolic dimension, and was expressed as a percentage. The access ports were entered and pressures obtained using externally calibrated transducers. The flow probe was connected to a digital flowmeter. From the digitized flow signal, stroke volume was computed on a beat-to-beat basis and averaged for a minimum of 25 ejections. Pulmonary and systemic vascular resistances were computed as the mean pressure divided by cardiac output multiplied by the constant 80 to convert to resistance units of $\text{dyne} \cdot \text{s} \cdot \text{cm}^{-5}$. Samples were drawn from the pulmonary artery and atrial catheters for measurement of oxygen saturation and hemoglobin content. The plasma samples were assayed for renin activity, endothelin concentration, and catecholamine levels.

EVALUATION

Results were presented as mean \pm SEM. Pairwise tests of individual group means were compared using Bonferroni probabilities.

MODIFICATIONS OF THE METHOD

Zhang et al. (1996) studied functional and bioenergetic consequences of post-infarction left remodeling in a porcine model. Proximal left coronary artery occlusion was used to generate a myocardial infarction in young pigs. The animals were then followed over sev-

eral months while remodeling of the left ventricle developed. Left ventricular wall thickness, ejection fraction, and wall stress were measured by MRI. Myocardial ATP, creatine phosphate, and inorganic phosphate levels were measured by spatially localized ^{31}P -NMR spectroscopy, and regional myocardial blood flow was measured with radioactive microspheres.

PROCEDURE

MRI Protocols

All MRI studies were performed on the standard Siemens Medical System VISION operating at 1.5 T. The animals were anesthetized with sodium pentobarbital. A catheter was placed into the femoral artery and advanced into the LV chamber for LV pressure recording. Animals then were placed on their left side in a Helmholtz coil with a diameter of 18 cm, which was used to improve signal to noise. To compute LV wall stress, the image acquisition was triggered by the LV pressure through the fluid-filled LV catheter. All of the imaging sequences were synchronized to the LV pressure trace. The electronic LV pressure signal was recorded and fed to a comparator set to a threshold level of 10% of the upslope of the LV pressure curve at the beginning of systole. The signal from the comparator was sent to a pulse former and then fed to the ECG port of the magnetic resonance system, where it was treated like the standard electrographic input to run the pulse sequences. Scout images were taken in the axial plane with a single-shot, ultrafast gradient echo sequence (McDonald et al. 1992; Wilke et al. 1993; Geiger et al. 1995). From the axial image, both horizontal and vertical long-axis images were obtained. By alternating back and forth several times, a true vertical long axis of the left ventricle was obtained. From the long-axis scout image, short-axis segmented cine turboflash slices were prescribed to cover the myocardium from apex to base. The double-oblique, short-axis turboflash images cover the heart from apex to base with a slice thickness of 10 mm, with no interslice gap.

MRI Cine Technique

The parameters of the segmented cine sequence were TR/TE/flip angle = 33 ms/6.1 ms/25 degrees with an FOV = 17.5 cm and a matrix of 87×128 (pixel size, $2 \text{ mm} \times 1.4 \text{ mm}$) and slice thickness of 7–10 mm (Atkinson et al. 1991). The sequence used segmented k-space acquisition such that three phase-encoded lines were gathered per cardiac phase per heartbeat. Total image acquisition required approximately 52 heartbeats for each slice location. The temporal im-

age resolution (data acquisition window) of this sequence was 33 ms per cardiac image. Each myocardial level took < 1.5 min to acquire, since two acquisitions were used and the average heart rate of the animals was 120 bpm. The average number of short-axis slices needed to image the entire myocardium from apex to base was six to eight. This 10-min protocol provided high signal-to-noise cine sequences covering the entire heart.

Spin-Echo Images

To obtain high-resolution anatomic heart images, multislice, single-phase spin-echo images triggered in the systolic phase were acquired to cover the entire heart. These images permitted the precise delineation of the extent of the scar region of the heart. Images were taken with a slice thickness of 5 mm and a FOV of 17.5 cm, resulting in a true spatial resolution of $2 \text{ mm} \times 1.4 \text{ mm}$ pixel size. The TR for this sequence equals the RR interval (500 ms) and the echo time TE was set to 30 ms. Total measurement time for an average of 10–14 slices was 5 min.

Image Analysis of the MRI Cine Studies

The imaging data were archived to optical disk and copied to a SUN SPARC 10 workstation for evaluation with the use of an automatic segmentation program (ImageView, Siemens Cooperate Research). The program is based on robust deformable models of endocardial and epicardial border segmentation of ventricular boundaries in cardiac magnetic resonance images. This segmentation technique has been combined with a user interface that allows one to load, sort, visualize, and analyze a cardiac study in < 20 min. The segmentation algorithm is based on the steepest descent as well as dynamic programming strategies integrated via multiscale analysis for minimizing the energy function of the resulting contour. The ventricular boundaries are used to construct a three-dimensional model for visualization and to compute hemodynamic parameters. Automatic segmentation of endocardial and epicardial boundaries was performed for calculation of ventricular volumes, EF, LV diastolic and systolic volumes, and absolute myocardial mass from multislice, multiphase magnetic resonance cine images. Starting with a user-specified approximate boundary or an interior point of the ventricle for one starting image in one slice, the algorithm generated automatic contours corresponding to the epicardium and the endocardium and automatically propagated them to other slices in the cardiac phase (spatial propagation) and to other phases for a given slice location (temporal propagation) of

the cardiac study. The observer then could make some manual corrections to the six or seven pairs of contours in the first column of the temporal-spatial matrix. Manual modifications generally were made on the apex and base levels.

EVALUATION

Mean LV wall thickness for each short-axis ring was averaged from three measurements of the remote zone (anterior wall and septum wall). The thickness of the scar was averaged from three measurements of the scar area. LVSA measurement in each slice was computed by subtracting the total area enclosed by the endocardium from that enclosed by the subepicardium; the resultant area was multiplied by the slice thickness to obtain the volume of each slice; the total LV mass volume was calculated by adding up the volumes of all the short-axis slices. The total LVSA was obtained by dividing the total LV wall mass volume by the mean of LV wall thickness of each slice. Similarly, the LVSSA was obtained by dividing the total scar volume, which was the sum of the scar volume of each short axis, by the mean of the scar thickness of each short axis. LV mass was computed by the total LV wall mass volume multiplied by 1.05 (specific gravity of myocardium) to calculate the LV mass. The LV end-diastolic volume (V_d) and end-systolic volume (V_s) of each slice were represented by the area enclosed by the endocardium. The total LV volume was computed by adding the volumes of all slices. LVEF was calculated by $100 \times (V - V_s) / V_d \%$. Interobserver and intraobserver errors for the calculations of LV mass and LV volumes have been shown to be <3 mg and 3 ml, respectively (McDonald et al. 1994). Meridional wall stress was computed from the LV pressure and simultaneously obtained short-axis view of LV MRI (LV cavity diameter and average thickness the remote LV wall) as described by Grossman et al. (1975).

Spatially Localized³¹P-NMR Spectroscopic Technique

Measurements were performed in a 40-cm-bore, 4.7-T magnet interfaced with a SISCO (Spectroscopy Imaging Systems Corporation) console. The LV pressure signal was used to gate NMR data acquisition to the cardiac cycle, while respiratory gating was achieved by triggering the ventilator to the cardiac cycle between data acquisitions (Robitaille et al. 1990). ³¹P- and ¹H-NMR frequencies were 81 and 200.1 MHz, respectively. Spectra were recorded in late diastole with a pulse repetition time of 6–7 s. This repetition time allowed full relaxation for ATP and P_i resonances and $\approx 90\%$ relaxation for the CP resonance (Zhang and

McDonald 1995). CP resonance intensities were corrected for this minor saturation; the correction factor was determined for each heart from two spectra recorded consecutively without transmural differentiation, one with 15-s repetition time to allow full relaxation and the other with the 6- to 7-s repetition time used in all the other measurements.

Radiofrequency transmission and signal detection were performed with a 25-mm-diameter surface coil. The coil was cemented to a sheet of silicone rubber 0.7 mm in thickness and $\approx 50\%$ larger in diameter than the coil itself. A capillary containing 15 μ l of 3 M phosphonoacetic acid was placed at the coil center to serve as a reference. The proton signal from water detected with the surface coil was used to homogenize the magnetic field and to adjust the position of the animal in the magnet so that the coil was at or near the magnet and gradient isocenters. This was accomplished with a spin-echo experiment and a readout gradient. The information gathered in this step also was used to determine the spatial coordinates for spectroscopic localization. Chemical shifts were measured relative to CP, which was assigned a chemical shift of -2.55 ppm relative to 85% phosphoric acid at 0 ppm.

Spatial localization across the LV wall was performed with the RAPP-ISIS/FSW method (Hendrich et al. 1991). Signal origin was restricted with the use of B_0 gradients and adiabatic inversion pulses to a column coaxial with the surface coil perpendicular to the LV wall. The column dimensions were 17×17 mm. Within this column, the signal was further localized using the B_1 gradient to five voxels centered about 45° , 60° , 90° , 120° , and 135° spin rotation increments. FSW localization used a nine-term Fourier series expansion. The Fourier coefficients, the number of free induction decays acquired for each term in the Fourier expansion, and the multiplication factors used to construct the voxels have been reported previously. The position of the voxels relative to the coil was set using the B_1 magnitude at the coil center, which was experimentally determined in each case by measurement of the 90° pulse length for the phosphonoacetic acid reference located in the coil center. Each set of spatially localized transmural spectra were acquired in 10 min. A total of 96 scans was accumulated within each 10-min block.

EVALUATION

Resonance intensities were quantified with the use of integration routines provided by the SISCO software. ATP γ resonance was used for ATP determination. Since data were acquired with the transmitter fre-

quency being positioned between the ATP γ and CP resonance, the off-resonance effects on these peaks were negligible. The numeric values for CP and ATP in each voxel were expressed as ratios of CP/ATP. P_i levels were measured as changes from baseline values (ΔP_i) with the use of integrals obtained in the region covering the P_i resonance.

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A.6.0.8

Cardiac Failure in Sheep

PURPOSE AND RATIONALE

Various methods have been used to induce cardiac failure in sheep: pressure overload after aortic banding (Aoyagi et al. 1993; Charles et al. 1996), volume loading after myocardial infarction (Charles et al. 2003), rapid ventricular pacing (Rademaker 1997, 2002b, 2005; Byrne et al. 2002; Moreno et al. 2005), coronary microembolization (Huang et al. 2004; Monreal et al. 2004).

Rademaker et al. (2002a) studied combined angiotensin converting enzyme inhibition and adrenomedullin in an ovine model of heart failure induced by rapid ventricular pacing.

PROCEDURE

Surgical Preparation

Coopworth ewes (38 ± 47 kg) were instrumented via a left lateral thoracotomy. Under general anesthesia (induced by 17 mg/kg thiopentone; maintained with halothane/nitrous oxide), two polyvinyl chloride catheters were inserted in the left atrium for blood sampling and left atrial pressure (LAP) determination; a Konigsberg pressure-tip transducer was inserted in

the aorta to record mean arterial pressure (MAP); an electromagnetic flow probe was placed around the ascending aorta to measure cardiac output (CO); a 7 French Swan-Ganz catheter was inserted in the pulmonary artery for infusions; and a 7 French His-bundle electrode was stitched subepicardially to the wall of the left ventricle for left ventricular pacing. All leads were externalized through incisions in the back. A bladder catheter was inserted per urethra for urine collections.

The animals were allowed to recover for 14 days before commencing the study protocol. During the experiments, the animals were held in metabolic cages, had free access to water and ate a diet of chaff and sheep pellets (containing 40 mmol/day sodium and 200 mmol/day potassium). A further 40 mmol of sodium was administered orally daily as NaCl tablets using an applicator.

Study Protocol

Heart failure was induced by 7 days of rapid left ventricular pacing (225 bpm) [15] and maintained by continuous pacing for the duration of the study. On four separate days with a rest day between each, the sheep received, in random order, a vehicle control (Haemacel), human adrenomedullin alone (50 ng/min per kg infusion for 3 h), an ACE inhibitor alone (captopril: 25 mg bolus + 2 mg/h infusion for 3 h), and both agents combined. Infusions were administered in a total volume of 60 ml via the pulmonary artery catheter, commencing at 10:00 hours.

Mean arterial pressure, left atrial pressure, cardiac output and calculated total peripheral resistance

(CTPR = mean arterial pressure/cardiac output) were recorded at 15-min intervals in the 1-h prior to infusion (baseline), and at 15, 30, 45, 60, 90, 120 and 180 min during both the 3-h infusion and post-infusion periods. Hemodynamic measurements were determined by on-line computer-assisted analysis.

Blood samples were drawn from the left atrium at 30 min and immediately pre-infusion (baseline), and at 30, 60, 120 and 180 min during the 3-h infusion and post-infusion periods. Samples were taken into tubes on ice, centrifuged at 3939 *g* for 10 min at 4°C and stored at either -20°C or -80°C before assay for immunoreactive (ir-) adrenomedullin, cAMP, plasma renin activity, angiotensin II, aldosterone, atrial natriuretic peptide, brain natriuretic peptide, endothelin-1, catecholamines and cortisol.

All samples from individual animals were measured in the same assay to avoid inter-assay variability. Plasma electrolytes and hematocrit were measured

in every sample taken. Urine volume and samples for the measurement of urine cAMP, sodium, potassium and creatinine excretion were collected every 1 h. Creatinine clearance was calculated as urine creatinine/plasma creatinine.

EVALUATION

Results are expressed as mean \pm SEM. Baseline hemodynamic and hormone values represent the means of the four and two measurements respectively made in the 1 h immediately pre-infusion. Statistical analysis was performed by repeated-measures ANOVA. Baseline data from all treatments were compared. Treatment- and time-related differences between all four study limbs were determined using a two-way ANOVA (treatment-time interactions are quoted in the text). Statistical significance was assumed when $P < 0.05$

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A.6.0.9

Cardiac Failure in Monkeys

PURPOSE AND RATIONALE

Several authors used monkeys for studies of cardiac failure. Hollander et al. (1977) investigated the role of hypertension in ischemic heart disease in the **cynomolgus monkey** with coarctation of the aorta. Sieber et al. (1980) studied cardiotoxic effects of adriamycin in **macaques** .

Various studies were performed by the group of Hoit and Walsh in **baboons** (Hoit et al. 1955a, 1955b, 1997a, 1997b; Khoury et al. 1996). Hoit et al. (1997a) studied the effects of thyroid hormone on cardiac β -adrenergic responsiveness in conscious baboons.

PROCEDURE

Animal Instrumentation

Adult male baboons (*Papio anubis*) weighing 21–30 kg were pre-instrumented for physiological monitoring in a lightly anesthetized, sedated state. Animals were pre-instrumented with a Konigsburg micromanometer and a polyvinyl catheter in the LV apex, miniaturized sonomicrometer pairs (3 MHz, 6 mm) across the LV anteroposterior minor axis, a polyvinyl catheter in the right atrium for central venous access, and pacing wires on the right atrial appendage. Wires and tubes were tunneled subcutaneously into the interscapular area for later use. Postoperative pain was reduced by the use of Buprenex (0.01 mg/kg i.m., q 6 h), and postoperative antibiotic (Monocid 25 mg/kg) was administered for 5 days to reduce the risk of infection. Baseline hemodynamic studies were performed after a minimum of 1 week for postoperative recovery.

Hemodynamic data acquisition and analysis The micromanometers and fluid-filled catheters were calibrated with a mercury manometer. Zero drift of the micromanometer was corrected by matching the LV end-diastolic pressure measured simultaneously through the LV catheter. The fluid-filled LV catheter was connected to a pre-calibrated Statham 23 dB transducer with zero pressure at the level of the mid right atrium. The transit time of ultrasound between the ultrasonic dimension crystals was measured with a multichannel

sonomicrometer (Triton Technology) and converted to distance assuming a constant velocity of sound in blood of 1.55 mm/ms.

The analog LV dP/dt signal was obtained on-line by electronic differentiation of the high-fidelity LV pressure signal. τ was derived from the high-fidelity LV pressure tracing by the method of Weiss et al. (1976), which assumes a monoexponential decay of LV pressure to a zero asymptote and has been shown to be directionally equivalent to other mathematical approaches for quantification of isovolumic pressure decay. τ is equal to the time in milliseconds for LV pressure to decay to $1/e$; thus, decreases in τ reflect improved isovolumic ventricular relaxation.

Fractional shortening of the LV minor axis was calculated as $(EDD-ESD)/EDD$, where EDD is LV end-diastolic dimension and ESD is LV end-systolic dimension. LV end-diastole was defined as the time in which LV dP/dt_{max} increased by ≥ 150 mmHg/s for 50 ms, and LV end-systole was defined as the time of the maximum ratio of LV pressure to LV minor-axis dimension. LV volumes were derived from minor-axis diameter (D) measurements: LV volume = $\pi/6(D)^3$.

V_{cf} was calculated as LV fractional shortening divided by LV ejection time; LV ejection time was defined as the time from peak positive to peak negative dP/dt .

Analog signals for high-fidelity and fluid-filled LV pressures, LV short-axis dimension, LV dP/dt , and the ECG were recorded on-line on a Gould multichannel recorder at 25 and 100 mm/s paper speed and digitized through an analog-to-digital board (Dual Control Systems) interfaced to an IBM AT computer at 500 Hz and stored on a floppy disk. Data were analyzed using an algorithm and software developed in our laboratory. Steady-state data were acquired over 5–10 s during spontaneous respiration and averaged.

Experimental Protocols

Hemodynamic studies were performed a minimum of 1 week after instrumentation and were repeated after 22–30 (26.8 ± 2.7) days of thyroid T_4 administration. Animals were tranquilized with Valium (1–5 mg) and ketamine (100 mg), and cholinergic blockade was achieved with atropine (0.4–0.8 mg i.v.); additional ketamine was administered as necessary, to a maximum cumulative dose of 40 mg/kg. Animals were atrially paced at a rate 40% to 50% greater than the control heart rate in order to obtain data at matched heart rates after thyrotoxicosis was produced.

Dobutamine Group

After hemodynamic stability was ensured and baseline data were recorded, intravenous dobutamine was infused at 5-min intervals at upwardly titrated rates of 2.5, 5.0, 7.5, and 10.0 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ to examine the effects of β_1 -adrenergic stimulation. The dose range of catecholamine for these studies was chosen to alter inotropic and lusitropic states without causing an untoward increase in heart rate. Steady-state hemodynamic measurements were made during minutes 4 and 5 of each infusion period. At each level, the pacemaker was briefly turned off to determine the effect of dobutamine on the heart rate.

Four of the animals in this group were studied with incremental pacing both before and after β -adrenergic blockade with esmolol (0.3 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ i.v.). The pacing protocol and the results from a larger group of animals studied before β -adrenergic blockade were detailed in a previous report. Briefly, atrial pacing was instituted at a rate above the intrinsic heart rate to avoid competing rhythms and was increased at 0.2-Hz increments until the critical heart rate was achieved. The critical heart rate was defined as the rate at which dP/dt_{max} and τ reached a maximum and minimum, respectively, during progressive increases in heart rate. We showed previously that hyperthyroidism significantly increases the critical heart rates for both dP/dt_{max} and τ .

The EC_{50} of dobutamine for LV dP/dt_{max} was determined by fitting log(dose)-transformed data to a sigmoidal relation with software from Graph Pad.

Terbutaline Group

Additional animals were chronically instrumented so that we could examine the effects of β_2 -adrenergic stimulation. One animal died suddenly after receiving thyroid hormone for 20 days. In the remaining three animals, the β_2 -adrenergic agonist terbutaline was infused both before and after production of the hyperthyroid state. Incremental doses of terbutaline (15 min/dose) were infused over a dosing range of 25 to 300 $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$.

Thyroid Function Tests

Thyroid function tests were performed before the baseline experiment in the euthyroid state and before the terminal experiment (within 24 h of the last dose of T_4) in the hyperthyroid state. T_3 radioimmunoassay, T_4 , and free T_4 levels were measured at each state.

EVALUATION

Paired mean data were compared by Student's *t*-test. The effects of thyroid status, catecholamine dose, and

β -blockade on hemodynamic and dimension variables were examined with repeated-measures ANOVA (SuperAnova, Abacus Concepts). When significant differences were found, group means were compared with contrasts. A value of $P < 0.05$ was considered significant. Unless specified, data are expressed as mean \pm SD.

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A.6.0.10

Cardiac Failure in Other Species

PURPOSE AND RATIONALE

Various species have been used to study experimental cardiac failure.

Breisch et al. (1984) studied the effects of pressure-overload hypertrophy in the left myocardium of young adult **cats**. Hypertrophy was induced by a 90% constriction of the ascending aorta.

Genao et al. (1996) recommended dilated cardiomyopathy in **turkeys** as an animal model for the study of human heart failure.

Do et al. (1997) studied energy metabolism in normal and hypertrophied right ventricle of the **ferret** heart.

Wang et al. (1994) studied Ca^{2+} handling and myofibrillar Ca^{2+} sensitivity in **ferret** cardiac myocytes with pressure-overload hypertrophy.

Bovine hereditary cardiomyopathy was recommended as an animal model of human dilated cardiomyopathy by Eschenhagen et al. (1995).

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A.6.0.11

Hypertrophy of Cultured Cardiac Cells

PURPOSE AND RATIONALE

Kojima et al. (1994), Komuro et al. (1990, 1991, 1993), Yamazaki et al. (1993, 1994, 1996) described a method to induce hypertrophy of cardiomyocytes by mechanical stress *in vitro*.

PROCEDURE

Primary cultures from cardiomyocytes are prepared from ventricles of 1-day-old neonatal Wistar Kyoto rats. According to the method of Simpson and Savion (1982), the cultures are treated for 3 days with 0.1 mM bromodeoxyuridine to suppress proliferation of non-myocardial cells. Elastic culture dishes ($2 \times 4 \times 1$ cm) are made by vulcanizing liquid silicone rubber consisting of methylvinyl polysiloxane and dimethyl hydrogen silicone resin using platinum as a catalyst. The bottom of the disc is 1-mm thick, and it is highly transparent because of no inorganic filler in either component. Cells are plated in a field density of 1×10^5 cells/cm² in culture medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Mechanical stress on cardiac cells is applied by gently pulling and hanging the dish on pegs. A 10% change in length of the dish results in an almost identical change in the length of the cell along a single axis (Kimuro

et al. 1990). Cardiocytes are stretched by 5%, 10%, or 20%. Drugs, e. g., an angiotensin-II receptor antagonist, are added 30 min before stretch.

For protein analysis, the silicone dishes are stretched for 24 h after 2 days of serum starvation and [³H]phenylalanine (1 $\mu\text{Ci}/\text{ml}$) is added for 60 min. At the end of each stress, the cells are rapidly rinsed four times with ice-cold phosphate-buffered saline and incubated for 20 min on ice with 1 ml of 5% trichloroacetic acid. The total trichloroacetic acid-insoluble radioactivity in each dish is determined by liquid scintillation counting.

For determination of mitogen-activated protein kinase, cardiomyocytes are lysed on ice and centrifuged. Aliquots of the supernatants of myocyte extracts are incubated in kinase buffer (25 mM/l Tris-HCl, pH 7.4, 10 mM/l MgCl_2 , 1 mM/l dithiothreitol, 40 $\mu\text{M}/\text{l}$ APT, 2 μCi [γ -³²P]ATP, 2 $\mu\text{M}/\text{l}$ protein kinase inhibitor peptide, and 0.5 mM/l EGTA) and substrates (25 μg myelin basic protein). The reaction is stopped by adding stopping solution containing 0.6% HCl, 1 mM/l ATP, and 1% bovine serum albumin. Aliquots of the supernatant are spotted on P81 paper (Whatman), washed in 0.5% phosphoric acid, dried and counted.

For determination of *c-fos mRNA*, Northern blot analysis is performed.

EVALUATION

Values are expressed as mean \pm SEM. Comparisons between groups are made by one-way ANOVA followed by Dunnett's modified *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The interesting approach to induce hypertrophy of cardiac cells *in vitro* has been used predominantly by one research group. Confirmation by other research groups including modifications of the mechanical procedures seems to be necessary.

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A.7

Positive Inotropic Activity (Cardiac Glycosides)

A.7.0.1

General Considerations

Biological standardization of cardiac glycosides was necessary as long as the drugs used in therapy were plant extracts or mixtures of various glycosides. They were standardized in units of an international standard. Some of the pharmacological methods used for these purposes and adopted by many pharmacopoeias have nowadays *historical interest* only. This holds true for the frog method and the pigeon method (Burn et al. 1950).

Particularly, the **frog method** was used for standardization. The method adopted by the U.S Pharmacopoeia X was the 1 h test. Healthy frogs (*Rana pipiens*) weighing 20–30 g were selected from the cold storage room. One hour before assay, their weight was recorded and they were placed in wire cages with a water depth of 1 cm. The doses of digitalis were calculated so that they approximated 0.015 ml/g body weight. Injections were made into the ventral lymph sac. One hour later, the animals were pithed and the heart removed and examined. Systolic arrest of the ventricle and widely dilated atrium indicated the typical result. Calculations of activity in terms of International Units were made from the percentage of dead animals in the test group versus those in the group receiving the international standard.

The **pigeon method** introduced by Hanzlik (1929) and adopted by USP XVII depends on the observation that intravenously injected cardiac glycosides have an emetic action in pigeons. In the original test, adult pi-

geons weighing 300–400 g are injected with a solution of the cardiac glycoside into a suitable wing vein in the axillary region. Vomiting occurring within 15 min is regarded as positive result. Two doses of test solution and standard are injected and percentage of vomiting pigeons registered. This 4 point assay allows calculation of ED_{50} values and of the potency ratio compared with the standard.

Modifications of other methods, such as the **cat method** introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngarden (1926), the **guinea pig method** described by Knaffl-Lenz (1926) and the **isolated cat papillary muscle** method introduced by Catell and Gold (1938) still being used for evaluation of synthetic cardioglycosides and other positive inotropic compounds are referenced in detail below.

Surveys on the evaluation of cardiac glycosides have been given by Bahrmann and Greef (1981), for the use of the isolated papillary muscle by Reiter (1981) and for other isolated heart preparations by Greef and Hafner (1981). Moreover, the influence on Na^+/K^+ -ATPase, an *in vitro* model specific for cardiac glycosides (Gundert-Remy and Weber 1981), is described.

The mechanisms of action have been reviewed by Scholz (1984) and Grupp (1987).

Analogous to antiarrhythmic agents, Feldmann (1993) proposed a classification system that categorizes inotropic agents according to their supposed mode of action:

Class I: Inotropic agents that increase intracellular cyloAMP, including β -adrenergic agonists and phosphodiesterase inhibitors,

Class II: Inotropic agents affecting sarcolemmal ion pumps and channels, in particular cardiac glycosides inhibiting Na^+/K^+ -ATPase,

Class III: Agents that modulate intracellular calcium mechanisms (no therapeutic inotropic agents in this kind yet available),

Class IV: Inotropic agents having multiple mechanisms of action

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A.7.1

In Vitro Tests

A.7.1.1

Ouabain Binding

PURPOSE AND RATIONALE

Cardiac glycosides can be characterized by their binding kinetics (association process, equilibrium binding, and dissociation process) on the ouabain receptor.

PROCEDURE

Heart sarcolemma preparations are obtained from rat or dog heart. From a canine heart or from rat hearts submitted to coronary perfusion myocytes are isolated by collagenase digestion. The isolated membrane fractions consist mainly of myocyte sarcolemma. [³H]ouabain with a specific radioactivity of about 20 Ci/mmol is incubated with ligands to be tested in 10 ml of binding medium consisting of 1 mM MgCl₂, 1 mM inorganic phosphate, and 50 mM Tris-HCl, pH 7.4 at 37°C for 10 min.

Association process: After temperature equilibration in the presence of either 10 or 100 nM

[³H]ouabain, 200 μg of membrane preparation are added to initiate the reaction. At various times, 4.5 ml are removed and rapidly filtered.

Equilibrium binding: At the end of the temperature equilibration carried out in the presence of increasing concentrations of [³H]ouabain ranging from 10 nM to 3 μM, 40 μg of membranes are added. After 30 min, duplicate aliquots of 4.5 ml are removed and filtered.

Dissociation process: Once equilibrium has been achieved under the experimental conditions used to study association, 10 ml of prewarmed Mg²⁺ plus P_i Tris-HCl solution supplemented with 0.2 mM unlabeled ouabain are added to initiate dissociation of [³H]ouabain. At various times, aliquots of 0.9 ml are removed and rapidly filtered.

All aliquots are filtered under vacuum on HAWP Millipore filters (0.45 μm) and rinsed three times with 4 ml of ice-cold buffer. The radioactivity bound to the filters and the specific binding measurements are determined.

EVALUATION

Kinetic parameters for the association and the dissociation process are calculated. The results of equilibrium binding are analyzed by Scatchard plots.

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A.7.1.2

Influence on Na⁺/K⁺ ATPase

PURPOSE AND RATIONALE

The enzyme Na⁺/K⁺ ATPase is the transport system for Na⁺ and K⁺ in the cell membranes. The membrane

bound enzyme couples ATP hydrolysis to the translocation of Na^+ and K^+ ions across the plasma membrane through a series of conformational transitions between the E_1 and E_2 states of the enzyme. The enzyme is a heterodimer consisting of a catalytic subunit (110 kDa) associated with a glycosylated β subunit (55 kDa). Three alpha (α_1 , α_2 , α_3) subunits have been identified by cDNA cloning. In the heart, enzyme Na^+/K^+ ATPase is the target of the positive inotropic glycosides. Therefore, it is of interest for the characterization of positive inotropic compounds. The test is based on the determination of phosphate generated from ATP under special conditions. Inhibition of bovine cerebral Na^+/K^+ ATPase prepared according to Schoner et al. (1967) is measured after addition of various concentrations of the test compound compared with those of the standard (Erdmann et al. 1980).

PROCEDURE

Solutions

1.00 ml 133 mM imidazole pH 7.3
 0.04 ml 160 mM MgCl_2
 0.02 ml DPNH (10 mg/ml)
 0.04 ml 310 mM NH_4Cl
 0.04 ml 100 mM ATP
 0.02 ml 40 mM phosphoenol-pyruvate
 0.05 ml pyruvate-kinase (1 mg/ml = 150 U/ml)
 0.04 ml lactate-dehydrogenase
 (0.5 mg/ml = 180 U/ml)
 0.20 ml 1 M NaCl
 0.01–0.02 ml bovine cerebral ATPase (depending on activity of the enzyme) up to 2.0 ml distilled water

Test

The enzyme activity is started by addition of the ATP solution at 37°C. After 4 min the inhibitor (various concentrations of the cardiac glycoside) is added. Na^+/K^+ ATPase activity is measured by a coupled optical assay. The reaction is continuously recorded and corrected for Mg^{2+} -activated ATPase by inhibition of Na^+/K^+ ATPase with 10^{-3} M ouabain.

EVALUATION

Inhibition of ATPase is measured after addition of various concentrations of the test compound. Dose-response curves are established and compared with the standard (k-strophanthin). Potency ratios can be calculated.

MODIFICATIONS OF THE METHOD

Brooker and Jelliffe (1972) and Marcus et al. (1975) described an *in vitro* assay based on displacement of

radiolabeled ouabain bound to ATPase by various glycosides. Another method is based on the inhibition of rubidium uptake into erythrocytes (Lowenstein 1965; Belz 1981).

Erdmann et al. (1980) prepared ($\text{Na}^+ + \text{K}^+$)-ATPase-containing cardiac cell membranes from rat hearts.

Maixent et al. (1987, 1991) described two Na, K -ATPase isoenzymes in canine cardiac myocytes as the molecular basis of inotropic and toxic effects of digitalis.

The effect of ouabain on Na^+/K^+ ATPase activity in cells of the human rhabdomyosarcoma cell line TE671 was studied by Miller et al. (1993) with a special equipment, the microphysiometer (McConnell et al. 1992).

CRITICAL ASSESSMENT OF THE METHOD

The *in vitro* methods being used for determinations of plasma levels of glycosides (Maixent et al. 1995) have been largely substituted by radioimmunoassays specific for individual glycosides. Nevertheless, the inhibition of Na^+/K^+ ATPase can be used as an indicator of activity of new semisynthetic cardiac glycosides.

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A.7.2

Tests in Isolated Tissues and Organs

A.7.2.1

Isolated Cat Papillary Muscle

PURPOSE AND RATIONALE

Isolated cardiac tissue has been chosen to study the decrease of performance after prolonged electrical stim-

ulation and during restoration of force under the influence of cardiac glycosides. Cattell and Gold (1938) described a method using cat papillary muscle.

PROCEDURE

Cats of either sex weighing 2.5–3 kg are used. The animal is anesthetized with ether and the thorax is opened rapidly. The heart is removed and a papillary muscle from the right ventricle is isolated and fixed in an organ bath containing oxygenated Ringer's solution at 36°C. One end of the muscle is tied to a tissue holder and the other one to a strain gauge. The muscle is stimulated electrically with 4–6 V, 2 ms duration and a rate of 30/min. The contractions are recorded on a polygraph. After 1 h, the muscle begins to fail and the force of contraction diminishes to a fraction of control. At this point, the cardiac glycoside is added to the bath, restoring the contractile force to levels approaching control. The standard dose is 300 ng/ml ouabain. The potency of natural and semisynthetic glycosides can be determined with this method. Catecholamines, like adrenaline (10 ng/ml) or isoprenaline (10 ng/ml), are active as well.

EVALUATION

The increase of contractile force is calculated as percentage of the predose level. Dose-response curves can be established using various doses.

CRITICAL ASSESSMENT OF THE METHOD

The use of isolated papillary muscle strips can be recommended for evaluation of inotropic compounds of various chemical classes.

MODIFICATIONS OF THE METHOD

Instead of cat papillary muscle the isolated left atrium of guinea pigs can be used (see Sect. A.1.2.6). For testing cardiac glycosides, the calcium content in the Ringer solution is reduced to 50%.

Andersom (1983) compared responses of guinea pig paced left atria to various positive inotropic agents at two different calcium concentrations (1.25 and 2.50 mM). Consistently good results were obtained at the lower calcium concentration with isoproterenol, ouabain, amrinone, and 3-isobutyl-1-methylxanthine.

Böhm et al. (1989) studied positive inotropic substances like isoprenaline and milrinone in isolated cardiac preparations from different sources. They used isolated papillary muscles from Wistar-Kyoto rats and from spontaneously hypertensive rats, but also human papillary muscle strips from patients with moderate

heart failure (NYHA II–III) and compared the effects with papillary muscle strips from patients with severe heart failure (NYHA IV). They recommended that new positive inotropic agents should be screened in human myocardial tissue from patients with heart failure.

Labow et al. (1991) recommended a human atrial trabecular preparation for evaluation of inotropic substances.

Böhm et al. (1989) tested positive inotropic agents in isolated cardiac preparations from different sources, e. g., human papillary muscle strips from patients with severe heart failure (NYHA IV), human papillary muscle strips from patients with moderate heart failure (NYHA II–III), human atrial trabeculae, isolated papillary muscles from Wistar–Kyoto rats, and isolated papillary muscles from spontaneous hypertensive rats. They suggested that positive inotropic effects should be screened in isolated myocardium from patients with heart failure.

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A.7.2.2

Isolated Hamster Cardiomyopathic Heart

PURPOSE AND RATIONALE

Special strains of Syrian hamsters develop cardiomyopathy. These animals can be used for evaluation of cardiotoxic drugs (see Sect. A.6.0.4).

PROCEDURE

Hamsters with cardiomyopathy (Bio 14/6) at the age of 50 weeks are used. Controls are normal Syrian hamsters (FIB hybrids) at the same age. The animals are pretreated with heparin (5 mg/kg i.p.) and 20 min later the heart is prepared according the method of Langendorff and perfused with heart Ringer solution under 75 mm H₂O hydrostatic pressure. The preparation is allowed to equilibrate in the isolated state for 60 min at 32°C with a diastolic preload of 1.5 g. The force of contractions is recorded isometrically by a strain gauge transducer on a polygraph, e. g., Heliscriptor He 19 recorder (Hellige GmbH, Freiburg, Germany). From these signals, the heart rate is measured by a chronometer. The coronary flow is measured by an electromagnetic flowmeter. Compounds are injected via the aortic cannula into the inflowing heart-Ringer solution.

EVALUATION

The contractile force and the coronary flow in hearts from diseased and normal animals is registered before and after application of the test drugs. Mean values and standard deviation are calculated before and after drug application and statistically compared using Student's *t*-test.

MODIFICATION OF THE METHOD

Jasmin et al. (1979) showed after prolonged *in vivo* administration beneficial effects of a variety of cardiovascular drugs, including verapamil, prenylamine, dibenamine and propranolol.

After chronic administration (4 or 12 weeks subcutaneously), Weishaar et al. (1987) found beneficial effects of the calcium channel blocker diltiazem, but not by the administration of digitalis.

In contrast, in the experiments of Ottenweller et al. (1987) hamsters treated orally with digoxin survived and showed significant amelioration of the pathological syndrome of heart failure.

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A.7.2.3

Potassium Loss from the Isolated Guinea Pig Heart

PURPOSE AND RATIONALE

Cardiac glycosides induce a net loss of potassium from cardiac tissue due to their inhibition of the Na⁺/K⁺ ATPase. Therefore, potassium is increased in the effluent of the isolated guinea pig heart. This phenomenon can be used as parameter for the activity of digitalis-like compounds (Lindner and Hajdu 1968).

PROCEDURE

The isolated heart of guinea pigs according to LAN-GENDORFF is prepared as described in Sect. A.3.1.1. The coronary outflow is measured by counting the drops of the effluent by a photocell. The effluent is collected in a funnel with a thin upwards shaped outlet allowing to withdraw small fluid samples for analysis by a flame photometer. A pump attached to a 4-way valve changes the samples to the flame photometer every 15 s in the following sequence: effluent Tyrode-solution from the heart, distilled water, Tyrode-solution used for perfusion, distilled water. The potassium content of affluent and effluent Tyrode-solution is compared and registered on a Varian-recorder. The difference is attributed to the potassium outflow from the heart. The dose-response curve is flat in the therapeutic range, much steeper in the toxic range.

EVALUATION

The following parameters are recorded and calculated:

- coronary flow [ml/min]
- contractile force
- potassium loss [mVal/min]

CRITICAL ASSESSMENT OF THE METHOD

A good correlation was found between the measured potassium loss and the positive inotropic effect of cardiac glycosides. The method is suitable for the quick

determination of efficacy of digitalis-like substances and facilitates the discrimination from other positive inotropic compounds like adrenaline.

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A.7.3

In Vivo Tests

A.7.3.1

Cardiac Toxicity in Cats (Hatcher's Method)

PURPOSE AND RATIONALE

The purpose of the method, originally introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngaarden (1926), was to establish “cat units” for cardiac glycoside preparations. Hatcher and Brody defined “the cat unit as the amount of crystalline ouabain which is fatal within about ninety minutes to a kilogram of a cat when the drug is injected slowly and almost continuously into the femoral vein”. Time to cardiac arrest after intravenous infusion of a solution with defined concentration of the standard was used as reference and the unknown solution of the test preparation compared with the standard. The method can be used for testing natural and semisynthetic glycosides.

PROCEDURE

Cats of either sex weighing 2–3.5 kg are temporarily anesthetized with ether. Anesthesia is maintained with 70 mg/kg chloralose given intravenously. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula is inserted. ECG is recorded from lead II. Then, intravenous infusion of the test solution is started. The endpoint is cardiac arrest which should be reached within 30–60 min by proper adjustment of the concentration of the infused solution.

MODIFICATIONS OF THE METHOD

Hatcher's original method has been modified by many authors. The method using **guinea pigs**, introduced by Knaffl-Lenz (1926) is in its essentials similar to the cat method.

Guinea pigs weighing 400–600 g are anesthetized with urethane (1.75 g/kg i.m.) The animal is secured

on a operating table and the trachea is cannulated. The jugular vein is cannulated for infusion of the test preparation. Cardiac arrest is recorded from ECG lead II.

Dogs and guinea pigs were used by Dörner (1955).

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A.7.3.2

Decay Rate and Enteral Absorption Rate of Cardiac Glycosides

PURPOSE AND RATIONALE

The basic principle of Hatcher's or Knaffl-Lenz's method is suitable to determine decay rates of cardiac glycosides. The decay of efficacy can be due to excretion or metabolic degradation of the glycoside.

PROCEDURE

Beagle dogs of either sex weighing 8–20 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula inserted. The vena femoralis is cannulated for continuous infusion of a defined concentration ($\mu\text{g}/\text{kg}/\text{min}$) of the test compound. ECG is recorded from lead II. The signs of first toxic effects, e. g., extra systoles, AV-block) are recorded. At this time, the infusion is terminated and the total dose/kg of the applied glycoside registered. After 4, 8, 12, or 24 h the infusion procedure is repeated. Within this period of time the glycoside administered with the first dose is only partially metabolized or excreted. Therefore, the dose needed for observation of ECG changes during the second infusion will be lower than in the first experiment.

EVALUATION

The dose required in the second experiment for induction of ECG changes is equal to the amount of metabolized or excreted glycoside. This value is expressed as percentage of the amount required in the first experiment and indicates the decay rate of the glycoside. Testing after various time intervals, the decay rate can be visualized graphically and half life times be calculated.

MODIFICATIONS OF THE METHOD

Rhesus monkeys have been used since their response to cardiac glycosides is more similar to that of man than that of dogs (Lindner et al. 1979).

The basic principle of Hatcher's or Knaffl-Lenz's method is also suitable to determine *enteral absorption of cardiac glycosides*. Again, for this purpose dogs are preferred instead of cats or guinea pigs. The dose to induce cardiac arrest is determined in 3–6 dogs. To other dogs, the same test compound is given intraduodenally at a dose below the intravenous lethal dose. Ninety or 180 min afterwards, the intravenous infusion with the same infusion speed and the same concentration of the test compound as in the previous experiments is started and time until cardiac arrest determined. The higher the duodenal resorption of the compound, the lower the dose of the intravenous infusion will be. For evaluation, the intravenous dose needed in the second experimental series (with enteral predosing) is subtracted from the dose of the first series (without enteral predosing) and indicates the amount of absorbed compound. This value is expressed as percentage of the value of the first series and indicates the absorption rate.

The efficacy and safety of a novel Na^+, K^+ -ATPase inhibitor has been tested in dogs with propranolol-induced heart failure by Maixent et al. (1992).

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A.8 Effects on Blood Supply and on Arterial and Venous Tonus

A.8.1

Models for Stroke and Multi-Infarct Cerebral Dysfunction

A.8.1.1

Cerebral Ischemia by Carotid Artery Occlusion in Mongolian Gerbils

PURPOSE AND RATIONALE

The Mongolian gerbil (*Meriones unguiculatus*) is extremely susceptible to carotid occlusion because of the peculiar anatomical occurrence of an incomplete circle of Willis without posterior communicating artery and a frequently rudimentary anterior communicating artery. Clamping of both carotid arteries induces a bilateral temporary brain ischemia (Levine and Sohn 1969; Bosma et al. 1981; Mršulja et al. 1983; Hossmann et al. 1983; Chandler et al. 1985). This pathological animal model allows the simulation of circulatory disturbances in the human brain. The hippocampus is one of the most vulnerable regions of the brain to ischemia and anoxia. The gerbil is known to develop selective neuronal damage in the CA1 sector of the hippocampus following brief periods of forebrain ischemia. This damage differs from conventionally described ischemic neuronal injury because of its slow development (Ito et al. 1975; Kirino 1982; Hossmann et al. 1983). The occlusion time can be varied allowing determination of various parameters, e. g. ischemia induced amnesia (see Sect. F.3.1.8).

PROCEDURE

Male Mongolian gerbils (strain: Hoe GerK jirds) weighing 48–88 g are randomly divided into groups (10–15 animals for each test and control group). Prior to testing, the animals are housed in a climate-controlled environment (21°C) with food and water available ad libitum. Fifteen minutes before surgery, the gerbils receive the test compound by oral or intraperitoneal administration. The control group is treated with vehicle alone.

The exposure of the common carotid arteries is performed under anesthesia with sodium pentobarbital (32 mg/kg i.p.), chloralhydrate (100 mg/kg i.p.) and atropine sulfate (0.8 mg/kg i.p.). The carotid arteries are isolated from surrounding tissue and a loop of un-

waxed dental floss is placed around each artery. A 2 cm length of double lumen catheter is passed from the level of carotid artery through the muscle layers of the dorsal surface of the neck. Each end of dental floss is threaded through a separate lumen, leaving a loose loop around the artery. Two days later, occlusion of each artery is produced by gently pulling the dental floss until the artery is completely occluded between the floss and the center wall of the catheter. Heifitz clips are placed on the floss against the exterior end of the tubing to maintain occlusion. After various intervals (5 to 30 min), the clips are removed and circulation is restored.

Complete bilateral occlusion of the arteries is confirmed by behavioral symptoms, i. e., depression of spontaneous motor activity, shallow and rapid respiration, and ptosis. Care is taken to avoid a drop of body temperature during any stage of the experiment. After experimental manipulations, animals are placed on a heating pad until complete recovery of motor activity.

Subjects are placed in individual observation glasses which are kept at a temperature of 29°C. They are observed for neurological symptoms (such as circling behavior, jumping and rolling seizures, opisthotonus, tonic convulsions, etc.) for 90 min.

After various intervals, the gerbils are sacrificed and their brains are removed.

EVALUATION

The following parameters are measured two hours after occlusion:

Degree of brain edema: water content (difference in weight of wet and dry brain)

Content of sodium and potassium. The hemispheres are separated and put on pre-weighted watch glasses to determine the wet weight. Then the hemispheres are dried in an open Petri dish for 2 days at a temperature of 95°C. After cooling off, the dry weight is noted. Sodium and potassium concentrations in the dried brain hemispheres are determined by flame photometry. The Na⁺/K⁺-ratio is calculated.

For histological examinations, the animals are sacrificed at 2 or 4 days after ischemia under ether anesthesia by decapitation. The brains are then removed and frozen in CO₂. Hippocampi are sectioned coronally with a cryostat at -14°C. The section thickness for Nissl and glial fibrillary acid protein (GFAP) staining is 20 μm and for the histochemical localization of calcium 30 μm.

Nissl staining and its quantitative evaluation. The sections are mounted by thawing on glass slides, Nissl

stained, and cover slipped with Permount. In order to standardize the histological procedure and to rule out the possibility that differences in the staining intensity were due to technical inconsistency, slides from control and experimental groups are processed together, stained for 5 min, and differentiated in a series of alcohols for 3 min each. The extent of hippocampal nerve cell damage (as reflected by cell loss and decreased stainability) is assessed by measuring the amount of Nissl-stained material in a predetermined representative region of the CA1 area with the aid of a guided densitometer (Leitz Texture Analysis System). The measuring field is $50 \times 500 \mu\text{m}$, fitting to the width of the CA1 soma layer (about $40 \mu\text{m}$).

Calcium localization. A modification of technique described by Kashiwa and Atkinson (1963) is used for the cytochemical localization of ionic calcium. The principle of the technique is that calcium complexes with a chelating agent producing an insoluble chromophore.

Stock solutions: Two solutions were used: a) glyoxalbis-(2-hydroxyanil) (GBHA), 0.4 g/100 ml absolute ethanol (2 ml 0.4% GBHA); and b) NaOH, 5 g/100 ml distilled water (0.3 ml 5% NaOH).

Staining procedure: First, cryostat sections are placed immediately in cold absolute acetone for rapid fixation for 5 min. Next, floating sections are transferred into 96% alcohol for 5 min and then transferred to staining solution for 3–4 min. Sections are then placed in 96% alcohol and mounted on glass slides. Because of quenching, it is necessary to view and photograph immediately.

GFAP (glial fibrillary acid proteinglial fibrillary acid protein) fluorescencemicroscopy. Following slide mounting, cryostat sections are fixed for 15 min in 3.5% formaldehyde solution in 0.01 M phosphate-buffered saline (PBS). The sections are incubated with mouse primary antibody against GFAP (Boehringer, Mannheim, FRG) for 30 min diluted 1:50 PBS. This antibody shows cross reactions also with GFAP from pigs and rats, indicating low species selectivity (Graber and Kreutzberg, 1986). The sections are rinsed in PBS and incubated to tetramethylrhodamine isothiocyanate (TRITC) specific for mouse immunoglobulin G (T-5393 from Sigma) diluted 1:50 in PBS. Control sections are incubated with PBS instead of primary antibody.

Measurements, expressed as extinction units per measuring field, are taken from three slides of each animal and averaged. Statistical analysis is done by Student's *t*-test.

MODIFICATIONS OF THE METHOD

“Sensitive” gerbils can be selected according to the method of Delbarre et al. (1988). In this method, pupil dilatation is obtained with atropine sulfate (1%) 20 min before anesthesia. Ocular fundus is examined with direct ophthalmoscope (Heine) before ligation and 5 min later. Only animals with an absence of retinal blood flow after ligation are considered as positive.

Using ^{31}P nuclear magnetic resonance spectroscopy, Sasaki et al. (1989) studied energy metabolism of the ischemic brain of gerbils *in vivo*.

An unanesthetized-gerbil model of cerebral ischemia was described by Chandler et al. (1985).

Using microdialysis, adenosine and its metabolites were measured directly in the brain of male gerbils by Dux et al. (1990). Two microdialysis probes (CMA/10, Carnegie Medicine, Sweden) were implanted stereotactically in the brain of the animals, one in the left dorsal hippocampus and one in the right striatum. The fibres were fixed to the cranium using dental wax. The dialysate was collected in 5 min intervals, and the concentrations of adenosine, hypoxanthine and inosine were determined by HPLC (Zetterström et al. 1982; Fredholm et al. 1984).

Kindy et al. (1992) measured glial fibrillary acid protein and vimentin on mRNA level by Northern blot analysis and protein content by immunoblot analysis in the gerbil neocortex, striatum and hippocampus after transient ischemia.

McRae et al. (1994) studied the effect of drug treatment on activated microglial antigens in hippocampal sections after ischemia in gerbils with cerebral fluid from patients with Alzheimer's disease and the amyloid precursor protein.

Nurse and Corbett (1996) found neuroprotection in gerbils with global cerebral ischemia after several days of mild, drug-induced hypothermia. The protection by the AMPA-antagonist NBQX may be due to a decrease in body temperature. A protracted period of subnormal temperature during the postischemic period can obscure the interpretation of preclinical studies.

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A.8.1.2

Forebrain Ischemia in Rats

PURPOSE AND RATIONALE

Smith et al. (1984) described a model of forebrain ischemia in rats induced by transient occlusion of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. This method has been used extensively (Nuglisch et al. 1990; Oberpichler et al. 1990; Rischke and Kriegelstein 1990; Kriegelstein and Peruche 1991; Nuglisch et al. 1991; Prehn et al. 1991; Rischke and Kriegelstein 1991; Seif el Nasr et al. 1992; Peruche et al. 1995).

PROCEDURE

Male Wistar rats weighing 250–300 g are anesthetized with 3.5% halothane and then connected to a Starling type respirator delivering 0.8% halothane and 30% O₂ in N₂O. The jugular vein and the tail artery are catheterized for withdrawal of blood and for monitoring blood pressure. Anticoagulation is achieved by intravenous heparin (200 IU/kg) application. Blood gases, blood pH, blood pressure, and blood glucose are measured 5 min prior to ischemia and 10 min after ischemia.

Halothane, but not N₂O, is discontinued and the rats are allowed to recover for 30 min. During this period, muscle paralysis is maintained with 5 mg/kg suxamethonium chloride, repeated every 15 min. After injection of trimethaphan camphor sulfonate (5 mg/kg), forebrain ischemia is induced by clamping of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. To prevent decay of intra-ischemic brain temperature, the environmental temperature is adjusted to 30°C by means of an infrared heating lamp. After 10 min of ischemia, the carotid clamps are removed and blood pressure is restored by re-infusing the shed blood. To minimize systemic acidosis, the rats receive intravenously 50 mg/kg NaHCO₃. The animals are removed from the respirator when they regain spontaneous respiration.

EVALUATION

Various parameters are used to evaluate the consequences of transient forebrain ischemia and the effectiveness of drug treatment:

- Local cerebral blood flow determination with the [¹⁴C]iodoantipyrine method (Sakurada et al. 1978),
- Histological assessment of ischemic cell damage in the hippocampus on day 7 after ischemia (Seif el Nasr et al. 1990; Nuglisch et al. 1990),

- Local cerebral glucose utilization using the [¹⁴C]deoxyglucose method described by Sokoloff et al. (1977),
- Quantitative analysis of the electrocorticogram (Peruche et al. 1995).

MODIFICATIONS OF THE METHOD

Kochhar et al. (1988) used two focal cerebral ischemia models in **rabbits**: a multiple cerebral embolic model by injection of microspheres into the internal carotid circulation and a spinal chord ischemia model by occluding the aorta for predetermined periods.

Gilboe et al. (1965) described the isolation and mechanical maintenance of the **dog** brain.

Andjus et al. (1967) and Krieglstein et al. (1972) described the preparation of the isolated perfused rat brain for studying effects on cerebral metabolism.

A cerebral ischemia model with conscious **mic**e was described by Himori et al. (1990).

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A.8.1.3

Hypoxia Tolerance Test in Rats

PURPOSE AND RATIONALE

The electrical activity of the brain is dependent on a continuous energy supply. Hypoxia is induced in test animals by inhalation of nitrogen. Marked hypoxia depresses cerebral metabolism resulting in an electrical failure of the brain. The procedure is used to investigate the ability of test compounds to antagonize the hypoxia-induced electrical failure of the brain by measuring the hypoxia-tolerance and the EEG recovery.

PROCEDURE

Male Sprague Dawley rats or stroke-prone rats weighing 250–300 g are used. They are anesthetized with hexobarbital sodium (100–120 mg/kg, i.p.) and surgically implanted with 2 epidural EEG-electrodes and a reference electrode to the parietal frontal cortex. After a minimum of a one week recovery period, testing can be started. The rats receive the test compound by intravenous or intraperitoneal administration. The control group is treated with vehicle alone. Thirty to 60 min after i.p. administration (immediately after i.v. administration) the animals are anaesthetized by

hexobarbital sodium at 100–120 mg/kg, i.p. When the stage of surgical anesthesia is reached, the animals are placed in a hypoxia chamber. EEG and ECG are recorded using a Hellige recorder.

Hypoxia is induced by inhalation of nitrogen (1200 l/h). On reaching an isoelectric EEG, the nitrogen inhalation is terminated and the animals are allowed to breathe room air. The recording is continued until EEG potentials can again be registered.

EVALUATION

The following parameters are measured in the test and control groups

- Hypoxia tolerance (HT): The time from the start of nitrogen inhalation until the onset of isoelectric EEG.
- Latency of EEG recovery: The time from the end of nitrogen inhalation until the onset of EEG potentials.
- The values of test and control groups are compared.

The percent change is calculated.

Statistics: Student's *t*-test by unpaired comparison.

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A.8.1.4

Middle Cerebral Artery Occlusion in Rats

PURPOSE AND RATIONALE

The permanent middle cerebral artery (MCA) occlusion technique in rats has been widely employed to evaluate various kinds of neuroprotective agents in cerebral ischemia (Ginsberg and Busto 1989). Focal cerebral infarction is achieved using a modification of the MCA occlusion model described by Tamura et al. (1981) and Pak et al. (1992).

PROCEDURE

Adult male Sprague-Dawley rats weighing 300–400 g are used in the experiments. The animals are anesthetized with a nitrous oxide-oxygen mixture (70:30%) containing 1.0 to 1.2% halothane. A mask is put on the nose and self-respiration is maintained during surgery, which takes around 20 to 30 min. The right femoral artery and vein are catheterized for monitoring blood pressure, blood sampling and administration of the

drug. Left MCA occlusion is performed via a subtemporal approach without removal of zygomatic arch or temporal muscle. Under high magnification of a surgical microscope, the left MCA is coagulated with a microbipolar coagulator from the olfactory tract to the most proximal portion of the MCA through a cranial window, about 3 to 4 mm in diameter, and cut afterward. Anesthesia is stopped just after MCA occlusion. The arterial catheter is removed soon after MCA occlusion, but the venous catheter is maintained for constant infusion of the drug by a swivel system (Harvard Apparatus, UK.). The animals are maintained normothermic (37°C) by a homeothermic system (Homeothermic Blanket System, Harvard Apparatus, UK.). During the surgery, mean arterial blood pressure (MABP) is recorded continuously (Model 7E polygraph, Grass, USA). Arterial gases and pH (178 pH/blood gas analyzer, Corning, USA) as well as hematocrit and blood glucose are measured twice, once about 15 min before MCA occlusion and the other just following MCA occlusion. Bilateral temporal muscle temperature (Therm 2250–1, Ahlborn Mess- und Regelungstechnik, Germany) is monitored during the surgery and several minutes before sacrifice.

Two, six, twelve and twenty-four hours after discontinuing anesthesia, each rat's level of consciousness and motor activity are evaluated using a grading scale of 0 to 3 (0: normal activity; 1: spontaneous activity+/-; 2: not arousable by tactile stimulation; 3: not arousable by painful stimulation, no spontaneous activity). Immediately before sacrifice, neurological status is examined using a grading scale of 0 to 3 (0: no observable deficit; 1: forelimb flexion; 2: decreased resistance to lateral push without circling; 3: the same status as grade 2, with circling). Four groups of rats are studied: vehicle-treated controls, and drug-treated animals at 3 different doses.

All the rats are re-anesthetized 24 h after MCA occlusion and sacrificed by intravenous injection of KCl. Immediately after sacrifice the brain is removed and frozen at -10°C for 10 min. The forebrain is cut into eight coronal slices by a rat brain slicer which are processed with the tetrazolium chloride (TTC) emulsion technique. Areas of brain not stained by TTC are drawn on a diagram at 8 preselected coronal levels of forebrain without knowledge of the experimental treatment. The areas of ischemic damage in the cerebral hemisphere, cerebral cortex, and caudate nucleus, drawn in the diagram are measured with a planimeter (KP-21, Koizumi, Japan) and integrated to determine the volumes.

REFERENCES AND FURTHER READING

Significance of the differences between the control and the treated groups is assessed by analysis of variance with subsequent inter group comparison by Student's *t*-test with Bonferroni correction. $P < 0.05$ is required for significance.

MODIFICATIONS OF THE METHOD

Hossmann (1982) reviewed the experimental models of cerebral ischemia.

Yamamoto et al. (1992) studied the inhibition of NO biosynthesis on the volume of focal ischemic infarction produced by occlusion of the middle cerebral artery in spontaneously hypertensive rats.

Shigeno et al. (1985) described a recirculation model following middle cerebral artery occlusion in rats. The trunk of the middle cerebral artery was isolated between the rhinocortical branch and the lenticulostriate artery and encircled with a loose-fitting suture of nylon thread. The thread was exteriorized through a small polyethylene catheter, which was previously introduced into the craniectomy site through a burr hole in the zygoma. The artery was occluded by retraction of the thread, which was then fixed with biological glue. Recirculation was achieved by cutting and removing the thread.

Bederson et al. (1986a) occluded the middle cerebral artery at different sites in six groups of normal rats and characterized the anatomical sites that produce uniform cerebral infarction. A neurological system was developed that can be used to evaluate the effects of cerebral ischemia.

Bederson et al. (1986b) evaluated the use of 2,3,5-triphenyltetrazolium chloride as a histopathological stain for identification and quantification of infarcted brain tissue after middle cerebral artery occlusion in rats.

Yang et al. (1992) found a reduction of Na,K-ATPase activity in the ischemic hemisphere shortly after middle cerebral artery occlusion in rats.

Du et al. (1996) induced transient focal cerebral ischemia in rats by a 90 min period of ligation of the right middle cerebral artery and both common carotid arteries.

Germano et al. (1987) found a decrease of stroke size and deficits in rats with middle cerebral artery occlusion after treatment with kynurenic acid, a broad spectrum antagonist of excitatory amino acid receptors.

Wu et al. (1999) reported that propentofylline attenuates microglial reaction in the rat spinal cord induced by middle cerebral artery occlusion.

Gotti et al. (1988) found a reduction of the volume of infarcted tissue due to occlusion of the middle cerebral artery in **rats** and **cats** after administration of NMDA receptor antagonists.

Hossmann and Schuier (1980) studied experimental brain infarcts in **cats** after occlusion of the left middle cerebral artery.

Retro-orbital occlusion of the middle cerebral artery in cats was performed by Sundt and Waltz (1966) and by Gotti et al. (1988).

Welsh et al. (1987), Backhaus et al. (1992) described focal cerebral ischemia in **mice** after permanent occlusion of the middle cerebral artery.

Hara et al. (1997) found a reduction of ischemic and excitotoxic neuronal damage by inhibition of interleukin 1 β -converting enzyme family proteases after occlusion of the middle cerebral artery in **mice** and **rats**. Nylon filaments were introduced from the carotid artery which were withdrawn after 2 h. One day later, the animals were tested for neurological deficits and the brains analyzed for infarct size and interleukin 1 β levels.

Huang et al. (1994) produced **knock-out mice** deficient in the neuronal isoform of NO synthase by targeted disruption of the neuronal nitric oxide synthase gene. In these mice, Hara et al. (1996) found reduced brain edema and infarction volume after transient middle coronary artery occlusion.

Nishimura et al. (1998) described an experimental model of thromboembolic stroke without craniotomy in **cynomolgus monkeys** by delivering an autologous blood clot to the middle cerebral artery. A chronic catheter was implanted in the left carotid artery in male cynomolgus monkeys. A 5 cm long piece of an autologous blood clot was flushed into the internal carotid artery with physiological saline. A neurological score was assigned at 0.167, 0.5, 1, 2, 4, 6, and 24 h after embolization. In the acute phase after embolization, typical behavior consisted of circling gait and moderate deviation towards the side of embolization, long-lasting and strong extensor hypotonia of the contralateral lower and upper limbs, and mild to severe incoordination. Contralateral hemiplegia was observed over the following 24 h. At 24 h the animals were sacrificed immediately after the last neurological scoring, and the cerebral vasculature was inspected for the location of the clot. The brain was then cut into 2 mm thick coronal sections. Cerebral infarction size and location were ascertained by the triphenyl-2H-tetrazolium chloride staining method. The lesions involved mostly the caudate nucleus, internal capsule, putamen and thalamus.

Salom et al. (1999) subjected female **goats** to 20 min global cerebral ischemia under halothane/N₂O anesthesia. An episode of transient global ischemia was achieved by occlusion of the two external carotid arteries and simultaneous external compression of the jugular veins by a neck tourniquet. A reperfusion period started when the occlusions were released, and it was monitored for 2 h.

De Ley et al. (1988) studied experimental thromboembolic stroke induced by injection of a single autologous blood clot into the internal cerebral artery in dogs by **positron emission tomography**.

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A.8.1.5

Photochemically Induced Focal Cerebral Ischemia in Rats

PURPOSE AND RATIONALE

Focal cerebral ischemia in rats can be induced by irradiation with intensive green light via a fibre optic through the skull after injection of the dye Rose Bengal. The resulting cerebral infarct can be studied for various parameters, such as infarct volume, water content, local cerebral blood flow and glucose utilization.

PROCEDURE

Male Sprague-Dawley rats (300–350 g) are used. Anesthesia is induced with 3% halothane in oxygen and is maintained with 1% halothane in oxygen applied via a face mask. A small incision is made in the skin over the right femoral vein and a thin catheter is

inserted. One ml of the dye Rose Bengal (in a concentration of 5 mg/ml of saline) is injected. A mid-line head incision is made and the right side of the skull is exposed. An intense green light, produced by a xenon lamp (75W, Zeiss, FRG) and then passed through a filter (wavelength 570 nm, Schott, Mainz, FRG) and a heat filter (Schott, Mainz, FRG), is directed on the skull at the level of the bregma for 15 min. The 3 mm diameter of the illuminated circle is determined by passing the light through a fibre optic (Schott, Mainz, FRG) while in close contact to the skull. The temperature of the skull underneath the fibre optic does not change during the time of illumination. At the end of the induction period, the temporary catheter is removed from the femoral vein which remains patent following the closure of the catheterization site with liquid suture (Histoacryl, Braun, Melsungen, FRG), and the incisions in the leg and head are likewise sutured after liberal application of local anesthetic. The anesthetic gas mixture is discontinued and the rats are allowed to recover consciousness in a warm environment until such times as the appropriate experiment is to be performed. Due to the non-invasive nature of this technique it is not possible to measure blood pressure or blood gases during the ischemic period. However, rectal temperature and plasma glucose concentrations are controlled.

Measurement of Infarct Volume

Osmotically controlled mini-pumps (Model 2ML1 Alzet, USA) are placed into the peritoneum of two groups of six male Sprague Dawley rats (body weight 300–350 g). The mini-pumps are fitted via thin polyethylene catheters to the femoral vein of the rats. Each pump contains either 2 ml of physiological saline or 2 ml of a solution of the drug to be studied. The animals are then given an ischemic insult as described in the previous section. Seventy-two hours after the induction of ischemia, the rats are sacrificed by decapitation and the brains removed and frozen at -50°C . Coronal sections ($20\ \mu$) are cut in a cryotome at -20°C , fixed in Haidenhain's Susa and stained with Cresyl Violet. The ischemic area on 90 sections is measured and the volume of ischemic change is then calculated using a linear trapezoidal extrapolation of the areas measured.

Measurement of Brain Water Content

Three groups each of six rats are used. One group receives no ischemic lesion (the illumination with green light is omitted from the experimental protocol) and two groups are lesioned as described above. One le-

sioned group receives the test drug orally at 15 min, 30 min, 1, 3 and 5 h after the induction of the ischemia or sham operation. The other group is treated with saline at the same time-points. Twenty-four hours later the rats are sacrificed by decapitation. The brains are rapidly removed and placed on a cutting block with 1 mm gradations. Two cuts are made 1 mm or less anterior and posterior to the lesion. The thick section so produced is then divided into left (non-lesioned) and right (lesioned) halves and placed in pre-weighed vials and the wet weights of the tissue samples are carefully measured. The tissue is then frozen in liquid nitrogen and then left under vacuum (less than 0.1 Torr) for 24 h. On removal the vials are sealed to prevent rehydration and reweighed to obtain the dry weight of tissue from which the water content (expressed as percentage of wet weight of tissue) is calculated.

EVALUATION

All data are presented as mean \pm SD of the mean. For left (contralateral to the lesion) against right (ipsilateral to the lesion) comparisons, a *t*-test with paired comparison was used ($P < 0.05$). Statistical differences between groups were calculated using the unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Boquillon et al. (1992) produced cerebral infarction in mice by intravenous injection of 10 mg/kg rose bengal, and by focal illumination of the intact skull surface for 3 min with a laser source, operating at 570 nm with power levels of 2, 5, 10, and 20 mW.

Matsuno et al. (1993) used a similar model to induce cerebral ischemia in rats based on middle cerebral artery occlusion by the photochemical reaction of rose bengal after irradiation with high intensity green light.

Stieg et al. (1999) studied neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat. An excellent correlation between infarct size determined by magnetic resonance imaging (MRI) and histopathological analysis in the same animals was found.

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A.8.1.6

Microdialysis and Neuroprotection Experiments After Global Ischemia in Rats

PURPOSE AND RATIONALE

Transient global ischemia can be induced in rats by electrocauterization of the vertebral arteries followed by clamping of the carotid arteries.

PROCEDURE

Male Wistar rats weighing 280–340 g are used. The rats are anesthetized with methohexital sodium (60 mg/kg i.v.) and the vertebral arteries are electrocauterized (Pulsinelli and Brierley 1979). The rats are fasted overnight and re-anesthetized on the following day with halothane and intubated. The femoral artery and vein are cannulated to allow blood sampling, blood withdrawal and recording of mean arterial pressure (MAP). Samples of blood are taken at regular time intervals and blood gas/acid base status is analyzed (Instrumentation Laboratory, 1306). Rectal temperature is measured with a thermistor and controlled at 37°C by means of a heating lamp. Four-vessel occlusion ischemia is induced for 20 min by bilateral carotid clamping followed by a period of reflow.

Microdialysis Experiments

The head of the rat is fixed in a stereotactic frame. The skin is incised over the head, pulled apart and a 3 × 3 mm hole is drilled through the cranium. A microdialysis probe (Sandberg et al. 1986) is implanted into CA1 region of the hippocampus (2.2 mm lateral and 3.8 mm dorsal to bregma, and the window of the dialysis membrane 1.4–2.9 mm below the cortical surface). The electroencephalogram (EEG) is measured continuously with a tungsten electrode attached to the dialysis probe. The probe is perfused at a rate of 2.5 µl/min with a modified Ringer solution. Dialysates are sampled every 10 min and analyzed for purines (Hagberg et al. 1987) and amino acids (Lindroth et al. 1985). One group receives the test drug i.p. 15 min prior to ischemia whereas the control group obtains

saline. The animals are followed during 20 min of ischemia and 2 h of reflow.

HPLC analyses are carried out using a reverse-phase C₁₈ column (Waters 10 µm µBondapak) with isocratic elution, a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine and hypoxanthine the mobile phase is 10 mM NH₄H₂PO₄, pH 6.0, 13% methanol.

Neuroprotection Experiments

During ischemic insult and for 20 min of reflow, the temperature of the temporalis muscle is controlled at 37°C. Immediately following ischemia the rats are divided into two groups. One group is treated with the test drug and the other with saline. A bolus injection is administered i.p. 15 min after ischemia and a mini-osmotic pump is implanted into the abdominal cavity which delivers the test drug for 7 days. Control animals receive a bolus of saline and mini-osmotic pumps filled with saline. Seven days later the rats are anesthetized with pentobarbital and perfusion-fixed with formol saline. The histological evaluation is done “blind”. The hippocampal damage is semiquantified according to the following scoring system:

0 = no damage

1 = scanty damage,

2 = moderate damage

3 = severe damage

4 = complete loss of pyramidal cells in the hippocampus.

EVALUATION

The purine and amino acid data are expressed as means ± SEM and differences are evaluated with the non-parametric Mann-Whitney test. The neuroprotective efficacy of the test drug is evaluated with two-tailed Student's *t*-test.

MODIFICATIONS OF THE TEST

In addition to the assessment of neuronal damage, Block et al. (1996) tested spatial learning of treated rats one week after 4-vessel occlusion in a Morris water maze.

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A.8.1.7

Hypoxia/Hypoglycemia in Hippocampal Slices

PURPOSE AND RATIONALE

The *in vitro* release of adenosine, inosine, and hypoxanthine from rat hippocampal slices can be determined with and without drug.

PROCEDURE

Male Sprague-Dawley rats weighing 150–275 g are decapitated, the hippocampi isolated and cut into 400 μ m thick slices which are placed into KRB (4°C) containing (mM) NaCl (118), KCl (4.85), MgSO₄ (1.15), KH₂PO₄ (1.15), NaHCO₃ (25), glucose (5.5), CaCl₂ (1.3) equilibrated with 95% O₂/5% CO₂. The incubation medium is brought up to room temperature over a period of 30 min. The KRB is then replaced with fresh medium and the slices are incubated for a further 30 min at room temperature, followed by 30 min at 37°C in fresh KRB. Following the initial incubations, slices are labelled for 45 min with ³H-adenine (5 μ Ci/ml) at 37°C. Two labelled slices are transferred into plastic cylinders which have nylon net bases and these, together with the slices, are placed into glass superfusion chambers. Slices are superfused at a flow rate of 0.5 ml/min with KRB at 37°C. After a 60 min wash, collection of 5 min fractions begins, which continues throughout the remainder of the experiment. A 1.25 ml aliquot of the

fractions is taken for determination of radioactivity using scintillation spectrometry (scintillation fluid: Picofluor 15). The remaining 1.25 ml is taken for HPLC analysis of purines and amino acids.

Hypoxia/hypoglycemia is induced by superfusion with KRB containing no glucose and 95% N₂ and 5% CO₂ for 35 min followed by recovery. All other procedures are as described above. The test drug is added to the perfusion fluid at an appropriate concentration. The 1.25 ml aliquots taken for HPLC analysis are pooled with two other aliquots (total 3.75 ml), lyophilized and concentrated 10 fold before analysis. Samples are analyzed for adenosine, inosine and hypoxanthine with HPLC. The radioactivity associated with each of these fractions is also determined by collecting the eluent from the column at the appropriate times. HPLC analyses are carried out using a reverse-phase C₁₈ column (Waters 10 μ m μ Bondapak) with isocratic elution, a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine and hypoxanthine the mobile phase is 10 mM NH₄H₂PO₄, pH 6.0, 13% methanol.

EVALUATION

Values for adenosine, inosine and hypoxanthine can be expressed in two ways: (1) release rate per slice (pmol/min \times slice); (2) percentage of the total amount of released radioactivity (% total ³H-label released). The purine data are expressed as means \pm SEM and differences are evaluated with the non-parametric Mann-Whitney test.

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A.8.1.8

Measurement of Local Cerebral Blood Flow and Glucose Utilization in Rats

PURPOSE AND RATIONALE

Cerebral glucose utilization can be determined using [¹⁴C]-2-deoxyglucose according to Sokoloff et al. (1977). Local cerebral blood flow using [¹⁴C]-iodoantipyrine is measured as described by Sakurada et al. (1978).

PROCEDURE

Animal Preparation

The experiments are performed on male Sprague-Dawley rats (260–300 g). Catheters are placed, under light halothane (1%) anesthesia, into the femoral vein and artery of each hind limb (for the measurement of mean arterial pressure, the sampling of arterial blood and the intravenous administration of drugs and radioisotopic tracer). The wound sites are then infused with local anesthetic, sutured, and protected by pads. The lower abdomen is covered by an elastic stocking, followed by a loose-fitting plaster coat. Anesthesia is discontinued and the rats are allowed at least two hours to recover before any further manipulations are performed.

Experimental Protocol

The rats are given an intravenous infusion (50 μ l/min) of either saline or the test drug dissolved in saline. This infusion is maintained throughout the measurement of local cerebral blood flow or local cerebral glucose utilization.

Local Cerebral Glucose Utilization

A full description of the method for measuring local cerebral glucose utilization using [14 C]-2-deoxyglucose has been published (Sokoloff et al. 1977). Five minutes after the administration of the test drug, the experiment is started with the intravenous administration of [14 C]-2-deoxyglucose (125 μ Ci/kg). Fourteen timed arterial blood samples are taken during the following forty-five minutes. These samples are centrifuged and the plasma is measured for glucose concentration (using an automated glucose analyzer) and [14 C] levels (by liquid scintillation counting). At the end of this period the rats are sacrificed by decapitation, the brain rapidly removed and frozen at -45° C. Twenty micron thick coronal sections are cut in a cryostat (-22° C) and autoradiograms are prepared by placing these sections in an array against Kodak SB-5 X-ray film along with pre-calibrated plastic standards range (55–851 nCi/g) for seven days in light-tight cassettes.

Local Cerebral Blood Flow

The autoradiographic measurement of local cerebral blood flow using [14 C]-iodoantipyrine is carried out as described by Sakurada et al. (1978). [14 C]-iodoantipyrine (125 μ Ci/kg) is administered fifteen minutes after the infusion of the test drug has commenced. In a period of sixty seconds, eighteen timed

arterial samples are collected in pre-weighed filter-paper discs from a free flowing arterial catheter. The discs are reweighed and the [14 C] concentration of each is measured by liquid scintillation counting. At the end of one minute, the rat is decapitated and autoradiograms are prepared in the same manner as for the measurement of local cerebral glucose utilization.

Densitometric Analysis of Autoradiograms

Tissue [14 C] concentrations were determined using a densitometer system (Zeiss, FRG) by reference to the images of the precalibrated standards. For each structure of interest, bilateral determination of optical densities are made on six different autoradiographic images in which the structure is best defined.

The mean optical density is used to calculate [14 C] concentrations. From this value, and the history of [14 C] in the blood, values of local cerebral blood flow and glucose utilization are obtained using the respective operational equations of these methods (Sakurada et al. 1978; Sokoloff et al. 1977).

EVALUATION

Groups of data are statistically compared by *t*-test with unpaired comparison using the BONFERRONI correction factor for multiple group analyses. Linear regression data for comparing cerebral blood flow (CBF) and glucose utilization (GU) undergo log transformation as recommended by McCulloch et al. (1982).

MODIFICATIONS OF THE METHOD

Ito et al. (1990) measured glucose utilization in the mouse brain by the simultaneous use of [14 C]2-deoxyglucose and [3 H]3-O-methylglucose.

High-resolution animal positron emission tomography was recommended by Magata et al. (1995) for noninvasive measurement of cerebral blood flow with 15 O-water and glucose metabolic rate with 18 F-2-fluoro-2-deoxyglucose.

The effect of ginseng pretreatment on cerebral glucose metabolism in ischemic rats using animal positron emission tomography (PET) with [18 F]-2-fluoro-2-deoxy-D-glucose ([18 F]-FDG) was described by Choi et al. (1997).

Hawkins et al. (1993) developed a method for evaluating tumor glycolytic rates *in vivo* with nude mice injected with 2-[18 F]fluoro-2-deoxy-D-glucose and a dedicated animal positron emission tomography scanner.

Positron emission tomography has been used with specific ligands for CNS imaging (de la Sayette 1991; Jones et al. 1991; Kung 1993).

Rogers et al. (1994) synthesized ^{18}F -labelled vesamicol derivatives to be evaluated in small animal positron emission tomography.

Hume et al. (1997) measured *in vivo* saturation kinetics of two dopamine transporter probes using a small animal positron emission tomography scanner.

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A.8.1.9

Cerebrovascular Resistance in Anesthetized Baboons

PURPOSE AND RATIONALE

Cerebral blood flow and cerebrovascular resistance can be measured by injection of inert radioactive gas (^{133}Xe) and evaluation of the ^{133}Xe clearance curve in anesthetized baboons.

PROCEDURE

Animal Preparation

The experiments are performed on baboons (*Papio* or *cynocephalus*) weighing 9–13 kg. Initial sedation with phencyclidine (10 mg i.m.) is followed by an intravenous injection of sodium thiopental (75 mg/kg). The animals are intubated to a positive pressure ventilator delivering 70:30% nitrous oxide and oxygen in open circuit. A continuous intravenous infusion of phencyclidine (0.01 mg/kg/min) is given throughout the course of the experiment. Suxamethonium (50 mg i.m.) is administered every 30 min in order to assist control of ventilation with the respiratory pump.

During the experiments, the end tidal concentration of CO_2 is continuously measured and the ventilating pump adjusted to maintain an arterial CO_2 tension (PaCO_2) of between 37 and 40 mm Hg for the control measurements. Arterial blood samples are taken during every measurement of cerebral blood flow (CBF) to measure PaCO_2 , PaO_2 , and pH by direct reading electrodes (Corning). Body temperature is maintained around 37°C by means of an electrically heated blanket and infrared heating lamps.

Catheters are inserted into the aorta via femoral arteries for the continuous measurement of arterial blood pressure and for the withdrawal of arterial blood samples. Both femoral veins are cannulated, one for the continuous infusion of phencyclidine and the other for the infusion of the test drug. A catheter is inserted into the right lingual artery so that its tip lays just distal to the carotid bifurcation. This catheter is flushed at regular intervals with heparinized saline to prevent platelet aggregation at the tip. In the studies with required intravenous administration of test drug, the other branches of the external carotid artery are ligated. Where the requirement is for intracarotid administration of test drug, another catheter is retrogradely advanced into the external carotid artery so that its tip lays next to the tip of the lingual catheter. This catheter is then attached to a constant-rate infusion pump (Sage Instruments). Heparinized saline is infused at a rate of 0.2 ml/min to act as a control for drug infusion.

A burr hole is made over the midline fissure and a catheter inserted into the sagittal sinus for the withdrawal of cerebral venous blood samples. The hole is sealed with plaster of Paris. The scalp and temporalis muscle are removed with diathermy down to the level of the zygomatic arch.

Measurement of Cerebral Blood Flow, Cerebral Oxygen Utilization, and Cerebrovascular Resistance

A collimated scintillation crystal is placed over the temporal region of the exposed skull on the right side and angled in such a way that viewed only brain and overlying skull.

Approximately 260 μCi of ^{133}Xe dissolved in 0.5 ml sterile heparinized saline (500 IU) is injected over 1 s into the catheter in the lingual branch of the carotid artery. The gamma-ray emission of the ^{133}Xe are detected by the scintillation counter attached to a photomultiplier. The pulses are amplified and subjected to pulse height analysis (peak 81 KeV \pm 8 KeV) to reduce Compton scatter before fed into a rate meter and scaler. The output from the rate meter is displayed on a chart recorder. Cerebral blood flow is calculated from the height/area equation (Høedt-Rasmussen et al. 1966). The formula used is

$$F = (H_2 - H_{10}) \times 100 / \lambda A_{10},$$

where F is CBF in ml blood per 100 g brain tissue per min; λ = brain tissue/blood partition coefficient for ^{133}Xe (the figure of 1.1 is used [Veall and Mallett 1966]); H_i = maximum initial height of the ^{133}Xe clearance curve in counts per min as taken from the

chart recorder; H_{10} = height of the clearance curve 10 min after the peak height in counts per s; A_{10} = total integrated counts over the 10 min of clearance as taken from the scaler and corrected for background activity over that period.

Cerebral oxygen consumption is measured from the product of the DBF and the difference in oxygen content between the arterial blood and cerebral venous blood sampled from the sagittal sinus. Blood oxygen is measured by a charcoal-fuel cell system (Lex-O₂-Con).

An estimate of cerebrovascular resistance is obtained by subtracting the mean sagittal sinus pressure from the mean arterial pressure and dividing this pressure difference by the CBF.

EEG Recording

Electroencephalographic readings are recorded bilaterally throughout the experiment. A series of holes are drilled in the calvarium 10 mm apart in two rows. Each row is 14 mm lateral to the sagittal suture. The holes are threaded to receive nylon screws in which silver-silver chloride ball electrodes are fixed loosely. The electrodes are positioned epidurally and the free ends are soldered to a multi-channel socket which is mounted on the calvarium with plaster of Paris.

Experimental Procedure

Following completion of the surgery, the animals are left undisturbed for 1 h. At least three control estimations of CBF and other parameters are made until steady conditions of flow, arterial blood pressure, and blood gas tensions are obtained.

Protocols

Intravenous administration. After stable control values have been established, the infusion is started. The CBF is measured at 5 min after the start and again at 25 min. The infusion is stopped 10 min after this flow period, giving a total infusion time of 35 min. Further flow measurements are made at 10, 30, and 50 min after stopping the infusion.

Intracarotid administration. After establishing control values, the intracarotid infusion of the test drug is begun. The CBF is measured at 10, 30, 50 and 70 min. The infusion is stopped 80 min after commencing, and post infusion measurements are made after 20, 40, and 60 min.

EVALUATION

Data are presented as mean values \pm SEM Evaluation of statistical significance is performed by means of Student's t -test with Bonferroni correction.

MODIFICATIONS OF THE METHOD

Kozniwska et al. (1992), Wang et al. (1992) measured cerebral blood flow in **rats** by intracarotid injection of ^{133}Xe .

Solomon et al. (1985) and Clozel and Watanabe (1993) induced cerebral vasoconstriction by injection of autologous blood in the cisterna magna of rats. Cerebral blood flow was measured with the radioactive microsphere technique.

Lin et al. (2003) described a model of subarachnoid hemorrhage-induced cerebral vasospasm in **mice**. Adult mice received injections of autologous blood into the cisterna magna. The diameters of large intracranial vessels were measured 1 h to 7 days after the subarachnoid hemorrhage. A diffuse blood clot was evident in both the anterior and posterior circulations. Vascular wall thickening, luminal narrowing, and corrugation of the internal elastic lamina were observed. Both acute (6–12 h) and delayed (1–3 days) phases of vasoconstriction occurred after subarachnoid hemorrhage. Overall mortality was only 3%. The model is recommended for screening of therapeutic candidates.

Delayed cerebral vasospasm was induced in anesthetized **dogs** by removal of 4 ml cerebrospinal fluid and injection of the same volume of fresh autologous arterial nonheparinized blood into the cisterna magna by Varsos et al. (1983) as a model of subarachnoid hemorrhage. The procedure was repeated on the third day and angiograms were taken of the vertebral-basilar vessels. The reduction of diameter of the basilar artery was taken as endpoint.

Imaizumi et al. (1996) produced experimental subarachnoid hemorrhage by intracisternal injection of arterial blood in **rabbits**. The degree of vasospasm and the effect of calcitonin gene-related peptide were evaluated angiographically by measuring the basilar artery diameter.

Inoue et al. (1996) produced experimental subarachnoid hemorrhage in **cynomolgus monkeys** by placing a clot around the internal carotid artery. A series of angiographic analyses were performed, before subarachnoid hemorrhage and on days 7 and 14 after treatment with calcitonin gene-related peptide to examine changes in the diameter of the ipsilateral internal carotid artery, middle cerebral artery, and anterior cerebral artery.

Hughes et al. (1994) adapted the ^{133}Xe clearance technique for simultaneous measurement of cutaneous blood flow in **rabbits** at a large number of skin sites within the same animal.

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A.8.1.10**Effect on Cerebral Blood Flow in Cats (Fluorography)****PURPOSE AND RATIONALE**

Cerebral blood flow in anaesthetized cats can be measured by fluorography. The heat transfer coefficient of

the brain can be measured with a probe representing an indirect blood flow value.

In this method, the measuring device consists of a thermo probe, which is attached to the tissue in order to record continually the heat transport (Hensel, 1956). The device depends on having an electrically heated part and an unheated reference point. The difference in temperature between the heated part of the device and the unheated reference point is a function of local blood flow. An increase in flow tends to lower the local temperature by carrying away the heat gain and vice versa.

PROCEDURE

Cats of either sex weighing 2.5–4.0 kg are anaesthetized by intraperitoneal administration of pentobarbital sodium (35 mg/kg) and intubated with a tracheal tube. The left femoral vein and the right femoral artery are cannulated for i.v. drug administration and determination of arterial blood pressure, respectively. The arterial cannula is connected to a Statham transducer P 23 Db.

For intraduodenal drug administration the duodenum is cannulated following laparotomy.

Before actually starting the experiment, the arterial blood gas concentrations are determined.

Animals are only used for further testing if they show normal blood gas concentrations. During the course of the experiment, blood flow, blood pressure and blood gas concentrations are regularly monitored.

The head of the animal is fixed in a stereotactic device. The skull cap and the dura are opened, the probes are placed on the surface of the cortex in the region of the marginal frontal gyrus, and the exposed brain is covered with moist swabs. The Fluvograph (Hartmann+Braun, Frankfurt) is used with the appropriate thermo probes.

To test the correct position of the thermocouple and the response of the animal, inhalations of carbon dioxide/ air or injections of epinephrine (adrenaline) are used, leading to a distinctive increase in cerebral blood flow. Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is administered.

Standard compound:

- pentoxifylline
 - 1 and 3 mg/kg (i.v. administration)
 - 10 and 30 mg/kg (i.d. administration)

EVALUATION

The percent change in the heat transfer coefficient is used as an indirect measure for the change of cerebral blood flow. Statistics: Student's *t*-test is performed by unpaired comparison.

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A.8.1.11

Effect on Cerebral Blood Flow and in Ischemic Skeletal Muscle in Rats (Laser-Doppler-Effect)

PURPOSE AND RATIONALE

The principle of the Laser Doppler effect is based on the fact that a laser light beam directed on tissue is scattered in static structures as well as in moving cells. Light beams scattered in moving red cells undergo a frequency shift according to the Doppler effect, while beams scattered in static tissue alone remain unshifted in frequency. The number of Doppler shifts per time is recorded as a measure for erythrocyte flow in a given volume. This means, that the direction of flow cannot be determined, but relative changes in micro circulatory blood flow can be recorded. The procedure can be used to detect test compounds that improve the blood supply of the brain or the flow of red blood cells in the ischemic skeletal muscle.

PROCEDURE

Male Sprague-Dawley rats weighing 300–500 g are anaesthetized with pentobarbital sodium (60 mg/kg i.p.). The trachea is exposed and intubated with a short tracheal tube to allow ventilation. The following vessels have to be cannulated: The femoral vein is cannulated for test drug administration. The femoral artery is cannulated for blood pressure recording and blood gas analysis.

Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further testing if they show normal blood gas concentrations (pa CO₂: 32–42 mm Hg; pa O₂: 70–110 mm Hg). The mean arterial blood pressure should not drop below 100 mm Hg. During the course of the experiment, blood flow, blood pressure and blood gas concentrations are regularly monitored.

For Cerebral Blood Flow

The head of the animal is fixed in a stereotactic device. After trepanation of the skull (opening 3 mm in diameter), the Laser Doppler probe is placed 1 mm above the surface of the brain. Values are measured with the Laser Doppler apparatus (Periflux F2, Perimed KB, Stockholm).

Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is infused. Following two administrations of the test compound, the standard compound is administered again. Duration of the effect is measured as half life in seconds.

For Peripheral Blood Flow

A small area of the femoral artery of the right hind limb is exposed and the Laser Doppler probe is placed 1 mm above the muscle surface. Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further proceeding if they show normal blood gas concentrations. During the course of the experiment, the blood pressure is recorded. The RBC flux is recorded continuously and after stabilization of the output signal, the femoral artery is occluded leading to underperfusion of the muscles of the right pelvic limb. At this stage, the test compound is administered by intravenous infusion for 10 min (0.05 ml/min).

Standard compound for cerebral blood flow is propentofylline (1 mg/kg, i.v.). The percent increase in blood flow produced by propentofylline ranges between 40% and 60%.

EVALUATION

The percent increase in blood flow after test drug administration is determined (compared to the value before drug administration).

Statistics: Student's *t*-test by unpaired comparison, test substance versus standard.

MODIFICATIONS OF THE METHOD

Iadecola (1992), Prado et al. (1992), Raszkievicz et al. (1992) measured the influence of nitric oxide on cortical cerebral blood flow in anesthetized rats by Laser Doppler flowmetry.

Benessiano et al. (1985) measured aortic blood flow with range-gated Doppler flowmeter in anesthetized rats.

Partridge (1991) measured nerve blood flow in the sciatic nerve of anesthetized rats with a Laser Doppler

Flowmeter after application of local anesthetics and of epinephrine.

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A.8.1.12**Traumatic Brain Injury****PURPOSE AND RATIONALE**

A major goal in research into mechanisms of brain damage and dysfunction in patients with severe head injury and in discovery of potential therapies is the development of a suitable animal model. While a variety of experimental techniques have been developed (Denny-Brown and Russell 1941; Gurdjian et al. 1954; Ommaya and Gennarelli 1974; Sullivan et al. 1976; Nilsson et al. 1977; Hayes et al. 1984; Marmarou et al. 1994; Mésenge et al. 1996), the most widely employed technique is fluid percussion, which produces brain injury by rapid injection of fluid into the closed cranial cavity (Sullivan et al. 1976; McIntosh et al. 1989, 1990; Faden et al. 1989; Dixon et al. 1991; Sun and Faden 1995; Petty et al. 1996; Laurer et al. 2000; Maegele et al. 2005). Most authors used rats, however, other species, such as cats (Sullivan et al. 1976; Hayes et al. 1983, 1984), rabbits (Lindgren and Rinder 1969) and mice (Hall et al. 1988; Mésenge et al. 1996) were employed.

PROCEDURE

Surgical Preparation

Male Sprague Dawley rats weighing from 400 to 500 g are anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). During surgical preparation and throughout the experiment, all wounds are infused with a topical anesthetic (lidocaine hydrochloride 2.0%). Catheters are inserted into the femoral vein for drug administration and into the femoral artery for blood pressure/blood gas monitoring. A 2.0 mm hollow female Leur-Loc fitting (to induce trauma) is rigidly fixed with dental cement to the animal's skull in a craniectomy centered over the left parietal cortex 5 mm from lambda, 5 mm from bregma, 4 mm from sagittal suture. The dura is left intact at this opening. Immediately following surgical preparation, a constant i.v. infusion of sodium barbital (15 mg/kg/h) is begun and maintained for the duration of the studies.

Drug Administration

Drugs or equal volumes of saline are administered through the femoral vein over 10 min by constant infusion beginning 15 min before trauma.

Fluid-Perussion Injury

The fluid-percussion device consists of a Plexiglas cylindrical reservoir, 60 cm long and 4.5 cm in diameter, bounded at one end by a Plexiglas, cork-covered piston mounted on O-rings. The opposite of the reservoir is fitted with a 2-cm-long metal housing on which a transducer is mounted and connected to a 5-mm tube (2 mm inner diameter) that terminates with a male Luer-Loc fitting. At the time of injury the tube is connected with the female Luer-Loc fitting that has been chronically implanted over the exposed dura of the rat. After the entire system is filled with 37°C isotonic saline, injury is induced by a metal pendulum which strikes the piston of the device from a predetermined height. The device produces a pulse of increased intracranial pressure of fairly constant duration (21–23 ms) by injecting various volumes of saline into the closed cranial cavity. Brief displacement and deformation of neural tissue results from the rapid epidural injection of saline. The magnitude of injury is regulated by varying the height of the pendulum, which results in corresponding variations of the intracranial pressure expressed in atmospheres. The pressure pulses are measured extracranially by a transducer and recorded on a storage oscilloscope.

EVALUATION

Behavioral Outcome

Posttraumatic deficits are evaluated at 24 h, 1 week and 2 weeks following trauma. Outcomes include forelimb flexion (right and left), lateral pulsion (right and left) and angle score (left, right and vertical position). Scores range from 0 (maximal deficits) to 5 (normal) for each task. By combining scores of all tests, a composite neuroscore is determined, ranging from 0 to 35 (Faden 1993).

Histopathology

At 2 weeks, following final neurological scoring, the rats are sacrificed by decapitation. The brain is removed, quickly frozen in isopentane and stored in a –80°C freezer until sectioning. Coronal brain sections are selected to span the longitudinal axis of the dorsal hippocampus between –3.2 and –3.8 Bregma. Sections (16 mm thick) are cut at –18°C in a microtome-cryostat and thaw-mounted onto chrome-gelatin rubbed microscope glass slides and kept at –80°C for histological study.

Sections are stained with Crystal violet. CA1 and CA3 pyramidal cells with a distinct nucleus and nucleolus are counted as viable neurons, in one reticle within CA3 and in three reticles (R1, R2 and R3) within the subfield of the hippocampus, in both right and left hemispheres. The number of viable neurons is counted twice at 400× microscope field.

An immunochemical method is used to detect glial fibrillary acidic protein (GFAP)-positive astrocytes in the hippocampus (Faddis and Vijayan 1988). Counting of the number of cells is done under 400× light microscopy in the dorsal CA1 subfield between medial and lateral regions.

Statistical Analysis

Neuroscores from forelimb flexion tests, lateral pulsion tests and angle board tests are statistically analyzed by non-parametric Mann-Whitney U-tests. Histological data are analyzed by one way ANOVA test, followed by Scheffe's test.

MODIFICATIONS OF THE METHOD

Shohami et al. (1995) tested the effect of a non-psychotropic cannabinoid which acts as a non-competitive NMDA antagonist on motor and memory functions after closed head injury in the rat.

Fox et al. (1998) developed a mouse model of traumatic brain injury using a device that produces controlled cortical impact, permitting independent manipulation of tissue deformation and impact velocity and

resulting in sustained sensory/motor and cognitive defects.

Tang et al. (1997) reported impairment in learning and memory in an experimental model of concussive brain injury in **mice**.

Bemana and Nago (1998) induced acute intercranial hypertension in **cats** by continuous inflation of an extradural balloon with physiological saline at a constant rate of 0.5 ml/h for 3 h. At this point, inflation was discontinued and the balloon remained expanded for an additional hour after which it was deflated.

A model of **traumatic injury to the spinal cord** was used by Springer et al. (1997). Female Long Evans rats weighing 200–250 g were anesthetized with pentobarbital and a dorsal laminectomy was performed to expose the spinal cord at thoracic level T10. The vertebral column was stabilized by clamping the column at vertebra 8 and 11. The New York University (NYU) impactor device was used which produces accurate and reproducible damage to the rat spinal cord (Gruner 1992; Basso et al. 1996). This device is a weight drop apparatus that uses optical potentiometers to record the movement of a 10-g impact rod and the vertebral column following impact and is connected to a PC that monitors rod and vertebral movements during impact.

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A.8.1.13**Cerebral Blood Flow Measured by NMRI****PURPOSE AND RATIONALE**

Over the past several years, magnetic resonance imaging (MRI) has become an established tool in the drug discovery and development process. The main advantages of MRI are its high resolution, non-invasiveness and versatility, which allow comprehensive characterization of a disease state and the effects of drug intervention. Recent advances now allow the application of this technique to the characterization of models of lung inflammation in rats and to the profiling of anti-inflammatory drugs. Repeated measurements can be carried out on the same animal, and time-courses of events can be easily assessed. Furthermore, the prospect of using MRI to detect non-invasively a sustained mucus hypersecretory phenotype induced by endotoxin brings an important new perspective to models of chronic obstructive pulmonary disease in animals. Importantly, it might be possible to extend the use of this technique to the clinical study of inflammation in the lung and the consequences of drug treatment (Beckmann et al. 2003, 2004).

Functional magnetic resonance imaging has been developed as pharmacological magnetic resonance imaging for pharmacodynamic assays and to establish brain-penetrating parameters (Leslie and James 2000). Although the regional cerebral metabolic rate of glucose is strongly increased during cerebral activity, the cerebral metabolic rate of O₂ is not increased in direct proportion. The result is that the relative uptake of O₂ from blood actually decreases. The veins and capillaries draining from the activated region are “arterialized” and their deoxyhemoglobin concentration is reduced. Deoxygenated and oxygenated hemoglobin have different magnetization properties; thus the changes in the ratio of these two entities can be detected by blood-oxygen-level-dependent (BOLD) magnetic resonance imaging. At least for studies in animals, these methods may be preferred to positron emission tomography (Cherry and Phelps 1996).

Petty et al. (2003) studied the *in vivo* neuroprotective effects of a systemically active antagonist of the NMDA receptor glycine site by magnetic resonance imaging in ischemic stroke in rats.

PROCEDURE***Transient Model of Focal Ischemia in Rats***

In the right common carotid artery was ligated. The transient model of middle cerebral artery occlusion was based on techniques described by Zea Longa et al.

(1989) and Belayev et al. (1996). By means of an operating microscope, the right common carotid artery was exposed through a midline neck incision. The right superior thyroid, occipital, and pterygopalatine arteries were ligated and cut. A poly-L-lysine-coated 3–0 nylon monofilament with a heat-blunted tip was inserted through the proximal external carotid artery into the internal carotid artery and pushed forward a distance of 19 or 20 mm from the carotid bifurcation, depending on the weight of the rat, so as to occlude the origin of the middle cerebral artery. After suture placement, the neck incision was closed and the animal was allowed to regain consciousness. Two hours following occlusion of the artery, the rats were tested on a standard neurological battery to confirm the presence of a neurological deficit. Animals that did not exhibit a forelimb flexion were excluded from further study. At this time, the rats were re-anesthetised and the intraluminal suture was completely withdrawn to restore the blood supply.

Magnetic Resonance Imaging (MRI) Measurements

Measurements were performed 30 min, 3 h, and 24 h after the onset of ischemia on a 7-T Bruker BIOSPEC experimental scanner (DBX; Bruker Medical, Ettlingen, Germany) with a 30-cm bore magnet and actively shielded gradient coils (200 mT/m; rise time < 80 μs).

A 72-mm resonator was used for *rf* transmission; signals were detected with a 35-mm inductively coupled surface coil placed over the skull of the animal. The *rf* coils were decoupled from each other – the transmitter coil actively and the receiver coil passively.

Using gradient-echo imaging, sagittal pilot scans were performed to ensure accurate positioning of the animal in the magnet. For this purpose, the coronal plane 5.9 mm posterior to the rhinal fissure was placed in the isocenter of the magnet, thus focusing on the center of the ischemic territory resulting from the middle cerebral artery occlusion.

For the determination of the temporal evolution of the ischemic lesion, two NMR imaging modalities were used. A field of view of 3.2 cm, a slice thickness of 1.5 mm, and an interslice distance of 2 mm were used for both sequences. Multislice packages were recorded by placing the center of the multislice imaging packet 5.9 mm posterior to the rhinal fissure.

Diffusion-weighted imaging was performed using a Stejskal–Tanner spin-echo sequence [echo time (TE) = 37.2 ms, repetition time (TR) = 2325 ms, eight slices] in six rats per group. To enable quantification of the apparent diffusion coefficient (ADC) of brain water, three *b* values were used (*b* = 50, 825,

and 1600 s/mm^2). ADC maps were calculated pixel-wise using the monoexponential model (Le Bihan et al. 1986).

Perfusion-weighted imaging was performed with an ultrafast version of the arterial spin tagging technique (Kerskens et al. 1996; Franke et al. 2000) in four rats per group. In independent experiments, three coronal slices were recorded, thus covering the central part of the ischemic lesion. Measurement parameters were: TE = 3.5 ms, TR = 7.4 ms, MATRIX = 128×64 , AVERAGE = 8. Each experiment consisted of two image acquisitions separated by a recovery period. During the first acquisition, blood flowing through the neck was adiabatically inverted (preparation TIME = 3 s; z-gradient = 5 mT/m; B1 FIELD = 150 mG; off-resonance FREQUENCY = 6000 Hz; mean preparation DISTANCE = 2.8 cm upstream from the imaging plane); in the second acquisition, inflowing spins were left undisturbed. Both phases were separated by a recovery period of 10 s. In each perfusion experiment, the two images suffered the same signal loss due to magnetization transfer effects but differed in the magnetization of the inflowing blood. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion. In the second acquisition, inflowing spins were left undisturbed. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion.

EVALUATION

Data were transferred to a PC and image analysis was carried out using the image processing software Scion Image for Windows (Scion Corporation, Frederick, Md., USA). Lesion volumes were calculated using ADC maps, as the ischemic lesion area was estimated by summing up all pixels with a relative ADC < 80% compared to the healthy, contralateral hemisphere (Hoehn-Berlage et al. 1995). Perfusion signal intensities are referred to the homologous contralateral regions and given as ratios of ipsilateral to contralateral values.

MODIFICATIONS OF THE METHOD

Edema following middle cerebral artery occlusion in spontaneously hypertensive rats was measured by magnetic resonance imaging (Seega and Elger 1993; Elger et al. 1994a). Magnetic resonance imaging was also used to determine the size of intracerebral hemorrhage in rats induced by stereotactic microinfusion of collagenase into the caudate putamen (Elger et al. 1994b).

Reese et al. (2000) visualized regional brain activation by bicuculline by functional magnetic resonance imaging. Time-resolved assessment of bicuculline-induced changes in local cerebral blood volume was achieved using magnetite nanoparticles as an intravascular contrast agent.

Pevsner et al. (2001) described a photothrombotic method of acute small stroke induction in rats with histopathologic and *in vivo* magnetic resonance imaging (MRI) observations from 3 to 6 h after irradiation, which is homologous to a human autopsy specimen. Utilizing 30 min of irradiation with minimal beam intensity (0.1 W/cm^2) cold white light in conjunction with 20 mg of intravenous (i.v.) rose bengal as a rapid infusion, small infarcts were induced photochemically in the frontal lobes of rats.

Using *in vivo* and *ex vivo* magnetic resonance imaging, Ohlstein et al. (2000) evaluated the effects of tranilast, an antiallergic drug, on neointima formation following balloon angioplasty of the rat coronary artery.

Swain et al. (2003) employed T-two-star (T_2^*)-weighted and flow-alternating inversion recovery (FAIR) functional magnetic resonance imaging to assess chronic changes in blood volume and flow as a result of exercise in rats. Prolonged exercise induced angiogenesis and increased cerebral blood flow in primary motor cortex.

Using MRI evaluation of brain damage, Banfi et al. (2004) demonstrated that pentoxifylline prevents spontaneous brain ischemia in stroke-prone rats.

Henderson et al. (2004) studied functional magnetic resonance imaging during hypotension in young and adult cats.

In a magnetic resonance imaging study Shirhan et al. (2004) found that spermine reduces infarction and neurological deficit a rat model of middle cerebral artery occlusion.

Paczynski et al. (2000) studied the effects of fluid management on edema volume and midline shift in a rat model of ischemic stroke. MRI were obtained 24 h after the onset of ischemia so that the ratio of hemispheric volumes ipsilateral and contralateral to the infarct and the extent of midline shift could be obtained.

Cash et al. (2001) evaluated the effectiveness of aminoguanidine as a neuroprotective agent in a rat model of transient middle cerebral artery occlusion using serial magnetic resonance imaging.

The MRI protocol consisted of three interleaved imaging regimens: proton density-weighted imaging (PDWI), T_2 -weighted imaging (T_2 WI) and diffusion-weighted imaging (DWI). Repetition time (TR) was 3 s for each of the three regimens and echo times (TE)

were 70 ms for the T₂WI and DWI and 28 ms for the PDWI. For DWI, diffusion-sensitizing gradients (b -value 590×10^{-3} s/mm²) were applied along the inferior to superior axis of the brain. Two averages were acquired per phase encode step. The images were collected at an in-plane resolution of 0.31×0.31 mm from 18 contiguous, 1-mm-thick slices, with a total acquisition time of 43 min.

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A.8.2

Peripheral Blood Supply

A.8.2.1

Perfused Hindquarter Preparation with Sympathetic Nerve Stimulation in Rats

PURPOSE AND RATIONALE

Perfusion of the hindquarter in rats with a constant flow rate allows the evaluation of the effect of drugs on the peripheral vascular bed. Since constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

PROCEDURE

Male Wistar rats weighing between 250 and 300 g are pre-treated with heparin (1000 units/kg i.v.) and anesthetized with pentobarbital sodium (50 mg/kg i.p.) The animals are intubated with a tracheal tube and positive pressure ventilation is maintained with a Harvard Rodent Respirator at 4–6 ml/stroke and 50 strokes/min. The right jugular vein is cannulated with polyethylene tubing for administration of drugs.

The lumbar sympathetic chain is isolated dorsal to the inferior mesenteric branches via an abdominal midline incision. The aorta is freed from the vena cava and two silk ligatures are placed around the aorta. The aorta is ligated and cannulated proximal as well as distally with polyethylene tubings. A short piece of rubber tubing is inserted at the distal end to allow intraarterial injections of drugs. Two “T” junctions allow the measurement of arterial pressure and perfusion pressure by Statham P 23 Db pressure transducers being recorded through a Hellige physiological recorder. From the proximal part of the aorta, blood is forced to its distal part by a peristaltic pump (Desaga) through a glass coil kept at 40°C. Flow rate is adjusted to produce a stable perfusion pressure as close to the systemic pressure as possible. After initial adjustment, flow rate is not altered for the remainder of the experiment.

Following perfusion pressure stabilization, the sympathetic chain is isolated and a small (1 mm wide, 2 mm long) curved bipolar electrode is placed around the nerve for electrical stimulation. Square-wave pulses from a Grass stimulator are used to activate the nerve with a constant current of 2.5 milliamps, supra-maximal voltage and varying frequencies of 5 ms duration.

A dose-response curve is established for norepinephrine by giving doses of 0.01 µg, 0.03 µg, 0.1 µg, 0.3 µg, 1.0 µg, and 3.0 µg intra-arterially and measuring perfusion pressure changes. Similarly, a frequency-response curve to nerve stimulation is established by stimulation at 3 Hz, 6 Hz, and 10 Hz for 30 s. Two pre-drug readings are taken to insure consistent responses.

A minimum of four animals is used for each test compound.

EVALUATION

The first predrug dose-response curves are compared with the second predrug, 5 min and 60 min postdrug dose-response curves. From regression equations for norepinephrine and nerve stimulation, mean responses and potency values with 95% confidence limits are calculated.

MODIFICATIONS OF THE METHOD

Folkow et al. (1970) perfused the hindquarters of spontaneously hypertensive rats and control rats at a constant rate of flow with an oxygenated plasma substitute in order to study the increased flow resistance and vascular reactivity. The hindquarters were isolated from the upper part of the body by standardized mass ligatures at identical levels until the aorta and the inferior caval vein provided the only intact circulatory connections between the two parts of each animal.

Thimm et al. (1984) described reflex increases in heart-rate induced by perfusing the hind leg of the rat with solutions containing lactic acid.

Thimm and Baum (1987) obtained spike recordings from chemosensitive nerve fibres of group III and IV of the rat nervus peroneus. Applications were performed either by perfusion of the circulatory isolated hindleg or by superfusion of the isolated musculus extensor digitorum longus.

Kitzen et al. (1978) used the perfused hind limb of the **dog** with sympathetic nerve stimulation for cardiovascular analysis.

Reitan et al. (1991) developed a near anesthetic-free isolated hindlimb model in the **dog** and studied the effects of halothane and atropine sulfate on vascular resistance.

Wieggershausen and Deptalla (1969) used the isolated perfused hindlimb of the **cat** to study the influence of local anesthetics on the vasoconstrictor actions induced by bradykinin, epinephrine and histamine.

Santiago et al. (1994) analyzed the responses to bradykinin in the hindquarters vascular bed of the cat.

Champion et al. (1996, 1997) analyzed the responses of human synthetic adrenomedullin, an analog of adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat.

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A.8.2.2

Effect on Peripheral Blood Flow in Rats

PURPOSE AND RATIONALE

Various methods exist to measure peripheral blood flow in rats, such as the microsphere technique, electromagnetic flowmetry and Doppler ultrasonic flowmetry.

Radioactive microspheres are used to calculate the distribution of blood from the heart to various organs and tissues before and after the administration of test compounds. In this method, microspheres are injected into the left cardiac ventricle. It is postulated that the first contraction of the cardiac muscle will expel these spheres into the circulation. Consequently the microspheres can be trapped in different organs according to

the organ's perfusion rate. It is not a primary screening method but it is a useful test for distinguishing compounds with blood flow altering activities.

The microspheres used are 14 μ in diameter. They are marked with isotopes. In this test, four different radioactive elements are used (Cr^{51} , Sr^{85} , Sc^{46} , Ce^{141}), allowing the determination of blood flow before dosing and after the administration of 3 different compounds or 3 different doses (of the same compound).

PROCEDURE

Male Wistar rats weighing 500–550 g are anaesthetized with pentobarbital. The trachea is exposed and intubated with a short endotracheal tube to allow ventilation. Prior to testing the jugular vein is cannulated for administration of test drugs. The carotid artery is cannulated and later on the catheter is passed retrograde into the ventricle. Ventricular pressure is recorded to assure the correct emplacement of the catheter tip in the ventricle. This catheter is connected to another catheter allowing the injection of microspheres into the left ventricle later on. The right arteria brachialis is cannulated and connected to a Hellige blood pressure recorder. During the course of the test, blood pressure will be measured continuously. The left femoral artery is cannulated and connected to an infusion pump. During the experiment blood will be withdrawn from this artery.

After these preparations the rat is allowed 15–30 min to recover. Before actually starting the experiment, the arterial blood gas concentration of each animal is measured. Animals are only used for the experiment if they show normal blood gas concentrations. During the following procedure blood pressure, ventricular pressure and the heart rate are continuously recorded. To determine baseline blood flow animals receive 0.2 ml vehicle/min over a 3 min period. In the 4th minute rats receive the first injection of microspheres (Cr^{51}). Simultaneously 0.5 ml/min blood is withdrawn from the femoral artery catheter for one minute, the pump thus being used as a reference organ. The animals are allowed 20 min to recover before the administration of drugs. The test compound is infused into the jugular vein at a rate of 0.2 ml/min for 3 min followed by injection of the second microsphere (Sr^{85}). The same procedure is repeated using the other two microspheres (Sc^{46} and Ce^{141}) following administration of the second and third test compound.

At the end of the experiment blood gas concentrations are measured. The animals are killed and their organs are removed. Usually blood flow is determined in the following organs:

- brain (right and left hemisphere; right hemisphere showing slight ischemia due to cannulation of carotids)
- cerebellum
- lungs
- heart
- kidney (right and left)
- skeletal muscle (right hind extremity)
- duodenum
- stomach
- spleen
- diaphragm
- adrenal gland (right and left)

To determine effects of test compounds on the blood flow in the underperfused skeletal muscle the same experiment can be performed with the right femoral artery being clamped. In this way effects of the test drug on the ischemic and normal skeletal muscle (left thoracic limb) can be compared in the same animal.

EVALUATION

The rate of blood flow/tissue at a certain time is determined by measuring radioactivity in the different tissues and comparing the results to that of the blood sample.

MODIFICATIONS OF THE METHOD

Blood flow in various peripheral organs, e. g., renal blood, flow can be measured with electromagnetic flowmeters (e. g., Transflow 601, Skalar Medical, Holland) or with Doppler ultrasonic flowmetry (Shaffer and Medvedev 1991).

Lappe et al. (1986) studied regional vascular resistance in conscious spontaneously hypertensive rats which were chronically instrumented with pulse Doppler flow probes to allow measurement of renal, mesenteric and hind quarters blood flow.

Hartman et al. (1994) validated a transit-time ultrasonic volume flow meter by simultaneous measurements with an electromagnetic flow metering method.

Lepore et al. (1999) used electron paramagnetic resonance to investigate the time course of nitric oxide generation and its susceptibility of nitric oxide synthase in ischemia-reperfusion injury to rat skeletal muscle *in vivo*. Total hind limb ischemia was applied for 2 h using a rubber band tourniquet method. At the end of ischemia the tourniquet was removed and the limb allowed to perfuse for various time intervals.

Beattie et al. (1995) measured carotid arterial vascular resistance in anesthetized **rabbits**. Carotid blood flow was measured by a Doppler flow probe

placed around the right common carotid artery. Dose-response curves of reduction of carotid arterial vascular resistance were constructed after injection of various doses of substance P-methyl ester via the right lingual artery. Intravenous injection of various doses of a selective tachykinin NK₁ receptor antagonist inhibited this effect dose-dependently.

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A.8.2.3

Effect on Peripheral Blood Flow in Anesthetized Dogs

PURPOSE AND RATIONALE

Effects on blood pressure have to be analyzed whether they are mediated by central, cardiac, or peripheral action. By injecting small doses of the test compound directly into a vascular bed, thus avoiding changes of central hemodynamics, peripheral vasodilating activity of a compound can be tested. This test is used to evaluate direct vasodilating or constricting activities of drugs *in vivo*-measurements of blood flow.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used. The dogs are premedi-

cated intravenously with heparin (bolus of 500 IU/kg and successive injections of 50 IU/kg every 30 min) and anesthetized by intravenous injection of thiobarbital sodium (0.5 mg/kg i.v.), chloralose (20 mg/kg i.v.) and urethane (250 mg/kg i.v.). Respiration is maintained with room air through a tracheal tube using a positive pressure respirator. Blood gas analysis is performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

To measure peripheral blood flow and to administer the test substance, a bypass is inserted into a femoral artery incorporating an electromagnetic flow probe and a port for injections. The other femoral artery is also equipped with a bypass used for the administration of a reference compound or a second test drug.

For recording of peripheral blood pressure and heart rate, one of the bypasses is connected to a pressure transducer (Statham P 23 BD).

All parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 20 min, the vehicle is administered (control), and 10 min later the test compound. Immediately after each administration, the port is flushed with physiological saline. Successive doses are administered after recovery to baseline values.

Readings are taken at times 0, 0.5, 1, 2, 5 and 10 min, and, if necessary, at additional 10 min-intervals following drug administration.

Standard compound:

- carbocromene 1 mg/kg
- Characteristics:
 - blood pressure
 - systolic, BPs
 - diastolic, BPd
 - heart rate, HR
 - peripheral blood flow, PF

EVALUATION

Changes in blood pressure, heart rate and peripheral blood flow at different times after drug administration are compared to vehicle control values obtained in the 10 min pre-drug period.

With $n > 2$, results are presented as mean \pm SEM. Statistical significance is assessed by means of the paired *t*-test. Scores are compared to the efficacy of

standard compounds for intensity and for duration of the effect.

MODIFICATIONS OF THE METHOD

Regional blood flow can be determined by the use of microspheres (Rudolph and Heyman 1967). The method is based on the principle that biologically inert microspheres will be trapped due their diameter in the microvasculature (Hales and Cliff 1977). The use of radioactive microspheres has some disadvantages (Buckberg et al. 1971). The use of fluorescent labeled microspheres for measurement of regional organ perfusion has been recommended (Glenny et al. 1993; Prinzen and Glenny 1994; van Oosterhout et al. 1995). Raab et al. (1999), Thein et al. (2000) described the automation of the use of fluorescent microspheres using a special sample processing unit. A Zymate-Robotic System (Zymark, Idstein, Germany) was modified to handle a special filtration device.

Ebara et al. (1994) measured renal blood flow in dogs after intrarenal arterial infusion of adrenomedullin.

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A.8.2.4**Effect on Peripheral Blood Supply Measured by Local Oxygen Pressure****PURPOSE AND RATIONALE**

Local oxygen pressure is directly related to oxygen supply to peripheral organs, e. g., muscle (Luebbers 1969; Kessler 1969). The local oxygen pressure (PO_2) is recorded directly on the muscle surface. In the following procedure the effect of test compounds on the local oxygen pressure (PO_2) of the normal and the ischemic skeletal muscle is determined.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The dog is anesthetized by intraperitoneal administration of pentobarbital sodium (Nembutal). Prior to testing, the following vessels have to be cannulated: The V. femoralis of the left pelvic limb is cannulated for administration of test compounds. The A. femoralis of the left pelvic limb is cannulated for blood pressure recording. The V. femoralis of the right pelvic limb is cannulated. During the course of this test blood will be withdrawn from this vein to monitor lactate concentrations.

Small areas of muscles of the right pelvic limb and the right thoracic limb are exposed. Muscle relaxation is induced by intravenous injection of 0.1 mg/kg alcuronium chloride (Alloferin) and maintained by i.p. administration of 0.05 mg/kg Alloferin at 30 min intervals. The trachea is exposed and intubated to assist the dog's respiration.

A PO_2 electrode is placed on the exposed muscle area of the right hind limb. After stabilization of PO_2 curves, the femoral artery of the right hind limb is occluded by putting a clip around the vessel. Muscle PO_2 drops rapidly. Following stabilization, test compounds are given by intravenous infusion for 10 min or by intraduodenal administration at this stage. The PO_2 of the non ischemic muscle is recorded simultaneously via a second electrode on the right thoracic extremity. The clip is removed after maximally one hour. This procedure can be repeated up to four times in one animal. Blood gas concentrations and pH are determined at the beginning and end of each experiment.

Standard compound:

- pentoxifylline

EVALUATION

The following parameters are determined:

- Maximal increase in PO_2 (mm Hg) after administration of test drug
- duration of effect by determining the half life

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A.8.2.5**Effect on Mesenteric Blood Flow in Rats****PURPOSE AND RATIONALE**

Blood flow in the mesenteric vascular bed *in situ* (Bhattacharya et al. 1977; Eikenburg 1984; Randall et al. 1989; Jackson and Inagami 1990) can be studied in rats in a way similar to that of the perfused hindquarter.

PROCEDURE

Wistar rats of either sex weighing 250–300 g are anesthetized with a combination of urethane (500 mg/kg i.v.) and sodium pentobarbitone (30 mg/kg). The abdomen is opened by a midline incision and a segment of the superior mesenteric artery is exposed by careful dissection of the surrounding tissue. Care is taken to avoid damage to the accompanying nerve terminals. One cannula is inserted into the carotid artery and the other into the superior mesenteric artery. Blood from the carotid artery is forced by a peristaltic pump (Desaga) into the superior mesenteric artery, using a glass coil kept at 40°C. Blood required to fill the tubing initially is obtained from donor rats. Heparin is administered intravenously to the animals prior to cannulation of the mesenteric artery. The blood pressure and the perfusion pressure are measured by Statham P 23 Db pressure transducers and recorded through a 2-channel Hellige recorder. The pump speed is initially

adjusted so that the perfusion pressure equals the systemic blood pressure. Intraarterial injections into the mesenteric vascular bed are made by puncturing the tubing going towards the periphery. Intravenous injections are made through a cannula inserted into the external jugular vein.

EVALUATION

Changes in the vascular resistance are measured by comparing perfusion pressure before and after drug administration. If constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

MODIFICATIONS OF THE METHOD

Reactivity in the mesenteric vascular bed can be tested in an isolated preparation (McGregor 1965; Kawasaki and Takasaki 1984; Laher and Triggler 1984; McAdams 1984; Foy and Nuhu 1985; Longhurst and Head 1985; Soma et al. 1985; Hsueh et al. 1986; Longhurst et al. 1986; Manzini and Perretti 1988; Nassar et al. 1988; Randall and Hiley 1988). The abdomen of anesthetized rats is opened and the superior mesenteric artery is separated from surrounding tissue in the region of the aorta. A cannula is inserted into the superior mesenteric artery at its origin from the abdominal aorta. The cannula is filled with heparinized Krebs solution. The ileocolic branch of the artery is tied off and the intestine separated from the mesentery by cutting close to the intestinal border of the mesentery. The cannulated artery and its vascular bed are dissected out and mounted in an organ bath. The preparation is perfused with oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. Perfusion pressure is recorded via a side arm of the arterial cannula using a Statham pressure transducer. The flow rate is adjusted to give a baseline perfusion pressure of 20–30 mm Hg. The test substances are infused into another side arm of the arterial cannula for 15 s using an infusion pump. After three stimuli with norepinephrine (1 µg) or potassium chloride (1 mg), the test drugs are infused followed by further stimulation. The inhibition of increase of perfusion pressure after test drugs is expressed as percentage of control.

Nuki et al. (1994) compared the vasodilating activity of chicken calcitonin gene-related peptide with human α -CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats.

The **rabbit** isolated arterially perfused intestinal segment preparation was used by Brown et al. (1983) as a model for vascular dopamine receptors.

Komidori et al. (1992) recommended the isolated rat mesenteric vascular-intestinal loop preparation as an excellent model for demonstrating resistance changes in isolated vascular beds while simultaneously measuring endogenous catecholamine overflow.

Pelissier et al. (1992) showed that perfusion with hypotonic solutions removed the endothelial layer in the isolated perfused mesenteric vascular bed of the rat, allowing the study of endothelial-dependent vascular responses.

Santiago et al. (1993) used the mesenteric vascular bed of the **cat** to study the inhibitory effects of the bradykinin receptor antagonist Hoe 140 on vascular responses to bradykinin.

The responses of adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat were compared by Santiago et al. (1995).

Chu and Beilin (1994) studied the mesenteric vascular reactivity which is reduced in pregnant rats after application of bradykinin and the bradykinin receptor antagonist Hoe 140.

Mulavi and Halpern (1977), Qiu et al. (1995) studied the mechanical and contractile properties of *in situ* localized mesenteric arteries in normotensive and spontaneously hypertensive rats.

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A.8.2.6

Effect on Pulmonary Blood Flow

PURPOSE AND RATIONALE

During controlled pulmonary blood flow, lobar arterial pressure can be measured in anesthetized cats (Lippton et al. 1984; Hyman et al. 1989; McMahan and Kadowitz 1993; deWitt et al. 1994).

PROCEDURE

Adult cats of either sex weighing 2.5 to 4.5 kg are sedated with ketamine hydrochloride (10–15 mg/kg i.m.) and anesthetized with pentobarbital sodium (30 mg/kg i.v.). The animals are fixed in supine position and supplemental doses of anesthetic are administered to maintain a uniform level of anesthesia. The trachea is intubated and the animals breath room air enriched with 95% O₂/5% CO₂. Systemic arterial pressure is measured from a catheter inserted into the aorta from a femoral artery, and intravenous injections are made from a catheter positioned in the inferior vena cava from a femoral vein.

For perfusion of the left lower lung lobe, a special designed 28-cm 6F triple-lumen balloon perfusion catheter (Arrow International, Reading, PA) is passed under fluoroscopic guidance from the left external jugular vein into the artery to the left lower lobe. The animal is heparinized by 1000 IU/kg i.v., and the lobar artery is isolated by distension of the balloon cuff on the perfusion catheter. The lobe is then perfused by way of the catheter lumen beyond the balloon cuff, with blood withdrawn from a femoral artery with a perfusion pump. Lobar arterial pressure is measured from a second port 5 mm from the cuff on the perfusion catheter. The perfusion rate is adjusted so that lobar arterial perfusion pressure approximates mean pressure in the main pulmonary artery. Left atrial pressure is measured with a 6F double-lumen catheter passed transeptally into the vein draining the left lower lobe. The catheter tip is positioned so that the left atrial pressure port on the distal lumen is 1–2 cm into the lobar vein and the second catheter port is near the venoatrial junction.

Lobar arterial pressure can be elevated to an high steady state level by the administration of *N*^ω-nitro-L-arginine^ω, followed by an intralobar infusion of the stable prostaglandin/endoperoxide analogue U-46619.

EVALUATION

Dose-response curves after administration of graded doses of drugs, e. g., decrease of lobar arterial pressure

after various doses of bradykinin, are established. The effects of antagonists, e. g. HOE 140, can be studied.

MODIFICATIONS OF THE METHOD

Liu et al. (1992) used a blood-perfused rat lung preparation to study pulmonary vasoconstriction or endothelium-dependent relaxation.

Byron et al. (1986) studied the deposition and airway-to-perfusate transfer of disodium fluorescein from 3–4 μm solid aerosols in an isolated perfused lung preparation of rats.

Mor et al. (1990) determined angiotensin-converting enzyme activity in the isolated perfused **guinea pig** lung.

Franks et al. (1990) used in Beagle **dogs** a single breath technique employing freon-22 as the soluble marker gas simultaneously with measurement of aortic blood flow by an electromagnetic flowmeter.

Tanaka et al. (1992) measured lung water content in dogs with acute pulmonary hypertension induced by injection of glass beads.

Drake et al. (1978) studied filtration characteristics of the exchange vessels in isolated dog lung by calculating the volume conductance with use of different components of the weight-gain curve following changes in capillary pressure.

Heaton et al. (1995) studied the effects of human adrenomedullin on the pulmonary vascular bed of isolated, blood perfused rat lung.

DeWitt et al. (1994), Lippton et al. (1994) investigated the effects of adrenomedullin in the pulmonary and systemic vascular bed of the **cat**.

Nossaman et al. (1995) compared the effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and the rat.

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A.8.2.7

Effect on Contractile Force of Ischemic Muscle

PURPOSE AND RATIONALE

Skeletal muscle is stimulated until it reaches its maximal force of contraction. This means that the muscle is forced to function at a level near exhaustion and has to use maximally the substrate supplied by the circulation. Then, the femoral artery is occluded, leading to underperfusion and a subsequent lack of substrate. As a consequence, the muscle's force of contraction decreases rapidly. Measuring the change in contractile force caused by drug administration reveals a drug's ability to restore ischemic muscle functions. In the following procedure, the drugs are tested for their effect on the force of contraction of the ischemic skeletal muscle.

PROCEDURE

Male Wistar rats weighing 400–450 g are anaesthetized by intraperitoneal administration of pentobarbital (Nembutal) (35 mg/kg). A tracheal tube is placed

to assist the rat's ventilation. The left carotid artery is cannulated for blood pressure recording and the left jugular vein is cannulated for the i.v. administration of test drugs. An incision is made to the skin of the right pelvic limb distal to the groin and the skeletal muscle is exposed down to the ankle. The skin is carefully trimmed away from the muscle to assure that contractions cannot be impaired by retraction of the skin. The major nerve supply is severed and a small length of the descending branch of the femoral artery is prepared free. The freely hanging muscle is attached to the force transducer (range 0–500 g, Z6, Rhema, Germany) and a resting tension of 50 g is placed on the muscle. To prevent dehydration, the skin is left attached to the muscle and the muscle is kept moist by the continuous drip of a 0.9% NaCl solution.

After these preparations, the rat is allowed to recover at least 30 min. Two needle electrodes are inserted into the muscle. Square impulses of 40 ms are generated with Stimulator 1 (Hugo Sachs Elektronik, Freiburg, Germany). The muscle is stimulated with a frequency of about 80 contractions per minute. The amplitude is increased gradually up to the muscle's maximal contractile force (usually between 2.0 and 3 mA). Following stabilization, the femoral artery is occluded with a clip for 5 min and subsequently reopened. After at least 15 min, test drugs are administered by intravenous infusion (0.075 ml/min) for 10 min. Five minutes after starting drug infusion, the artery is clamped again (for 5 min) while drug infusion is still going on. The force of contraction is continuously recorded. After declamping the artery, the rat is allowed to recover for at least 30 min before the whole procedure is repeated with another test drug. In this way, 3 different compounds can be tested in the same animal.

Standard compound:

- pentoxifylline

EVALUATION

The following parameters are measured:

- the percent inhibition of contractile force before drug administration (artery being clamped)
- the percent inhibition of contractile force after drug administration (artery being clamped)

The percent increase in contractile force after drug administration is calculated.

CRITICAL ASSESSMENT OF THE METHOD

An attempt is made to measure not only the effects of the drug on vasculature tonus but also on muscle metabolism.

MODIFICATIONS OF THE METHOD

Weselcouch and Demusz (1990) studied drug effects in the ischemic hindlimbs of **ferrets**. The hindlimb was stimulated to contract isometrically via supramaximal electrical stimulation of the sciatic nerve. Ischemia was induced by partial occlusion of the abdominal aorta. Pentoxifylline attenuated the loss of function in a dose-related manner.

Okyayuz-Baklouti et al. (1992) studied the functional, histomorphological and biochemical changes in atrophying skeletal muscle using a novel immobilization model in the rat.

Le Tallec et al. (1996) reported the effects of dimethylformamide on *in vivo* fatigue and metabolism in rat skeletal muscle measured by ³¹P nuclear magnetic resonance spectroscopy.

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A.8.2.8

Effect on Perfusion of Rabbit Ear (Pissemiski Method)

PURPOSE AND RATIONALE

The procedure was described as early as 1914 by Pissemiski, based on experiments of Krawkow (1913) in fish gills. It can be used to elucidate vasoactive properties (both vasoconstrictive and vasodilating) of

compounds. The isolated rabbit's ear is used to determine the effect of test compounds on its perfusion rate. Administration of norfenephrine induces vasoconstriction leading to a decrease in perfusion rate. A compound with vasodilatory properties will inhibit the norfenephrine induced fall in perfusion rate whereas a vasoconstrictor will potentiate this effect.

PROCEDURE

A rabbit of either sex weighing 1.5–3 kg is sacrificed by CO₂ narcosis and its ears are severed immediately. The ear is placed on a glass disc, the posterior auricular artery is exposed and cannulated. The cannula is connected to a tubing with a T-branch allowing the infusion of different solutions. Ringer's solution, kept at room temperature, is infused under 40 cm water column pressure via the cannula. The perfusion flow volume is recorded using a time ordinate recorder and a CONDON tipper or a photoelectric drop counter.

Prior to drug administration, the pH of the Ringer solution (containing test compound) must be determined. If the pH is greater than 8.5 or smaller than 6.5, it should be adjusted by adding a diluted NaOH- or HCl-solution.

Testing for Vasodilatory (Norfenephrine Antagonistic) Activity

Norfenephrine is infused at a concentration of 0.5 µg/ml until the maximal contraction is reached. The test compound is prepared in Ringer's solution at a concentration of 100 µg/ml. A volume of 30 ml is infused via the cannula over a 15 min period under constant pressure. The change of perfusion rate is determined. If there is a positive response (increase in perfusion rate), the test may be repeated using lower concentrations. If there is a negative response (further decrease in perfusion rate), the compound can be tested for vasoconstrictive activity.

Testing for Vasoconstrictive Activity

This test is repeated as described above without administration of norfenephrine.

Standard compounds:

- as vasodilator
 - dihydralazine
 - theophylline
 - pentoxifylline 100 µg/ml
- as vasoconstrictor
 - norfenephrine (Novadral)

EVALUATION

Testing for Vasodilatory Activity

The perfusion rate of the ear vessel is determined during the course of the test:

R = perfusion rate of vehicle perfused vessel

RN = perfusion rate of norfenephrine constricted vessel

RNP = perfusion rate of norfenephrine constricted vessel following compound administration

The percent inhibition of norfenephrine induced decrease in perfusion pressure is calculated using the following formula:

$$\% \text{ inhibition} = \frac{(RN - RNP)}{R - RN} \times 100$$

Testing for Vasoconstrictive Activity

The normal perfusion rate (ear vessel perfused with Ringer's solution) is taken as 100%. The percent inhibition of perfusion rate following compound administration is determined.

MODIFICATIONS OF THE METHOD

Schlossmann (1927) used the isolated rabbit ear preparation according to Pissemski for determination of the adrenaline content in blood.

De la Lande and Rand (1965), de la Lande et al. (1967) described a method of perfusing the isolated central artery of the rabbit ear. Small segments of the artery, taken from the base of the ear, were perfused at a constant rate with Krebs solution at 37°C. To enable drugs to be applied either to the intima or the adventitia, the artery was double cannulated so that the intraluminal and extraluminal perfusion media did not mix. Constrictor responses were measured by the maximum rise in perfusion pressure.

Steinsland et al. (1973) studied the inhibition of adrenergic transmission by parasympathomimetics in the isolated central ear artery of the rabbit. Perfusion was performed at a constant flow rate with Krebs' solution and perfusion pressure was recorded with a transducer.

Allen et al. (1973) incubated isolated segments of rabbit ear artery with (³H)-(-)-noradrenaline and measured the amount of tritium released into the luminal perfusate and into the extraluminal superfusate.

Budai et al. (1990) used isolated proximal 3–4-cm segments of the rabbit ear artery or rat tail artery in a low volume perfusion-superfusion system for measurement of transmitter release from blood vessels *in vitro*.

Miyahara et al. (1993) used arterial rings rabbit ear arteries *in vitro* which were contracted by perivascular nerve stimulation, or 5×10^{-7} noradrenaline or high potassium (29.6 mM) solution. High doses of dexamethasone or clobetasol-17-propionate decreased the amplitude of contractions. Furthermore, the authors performed *in vivo* experiments in albino rabbits, whereby the fur was removed from the distal parts of the ear by applying a depilatory cream at least 24 h before the experiments. The apical regions of the ear were then stripped with adhesive tape 7 times to remove the keratinous epidermal layer. The rabbit was anesthetized and the experimental parts of the ear were placed under a high resolution magnifying camera and immobilized using bilayer adhesive tapes. The vascular reactions induced by topical application of corticosteroids were recorded chronically using videotapes.

Aoki and Chiba (1993) described a method for separate intraluminal and extraluminal perfusion of the **basilar artery** in dogs. A polyethylene roof was designed to cover the canine basilar artery so that an extraluminal superfusion stream could pass over the artery that simultaneously received an intraluminal perfusion.

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A.8.2.9

Effect on Venous Tonus In Situ in Dogs

PURPOSE AND RATIONALE

Veins can be classified into two groups: those that respond and those that do not respond to epinephrine, acetylcholine and sympathetic nerve stimulation. As studies in dogs have shown (Rice et al. 1966) the reactive veins have a considerable amount of smooth muscle whereas the nonreactive ones lack any appreciable amount of smooth muscle fibres in the tunica media. A special preparation allows the registration of localized venous vasoconstriction.

PROCEDURE

Dogs weighing 20–30 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The trachea is cannulated and the dog ventilated with a respiration pump. The femoral vein is cannulated for systemic injections. After administration of 5 mg/kg heparin sodium i.v., the saphenous vein and the femoral artery are cannulated. The venous cannula is placed approximately 1 cm distal to its junction with another vein. After the non-perfused branch of the junction is ligated, constant blood flow is maintained from the femoral artery by using a Sigmamotor pump. The flow is adjusted so that a normal physiologic pressure in the vein is maintained. Perfusion pressure is measured between the pump and the vein so that any changes in pressure reflect changes in venous resistance. The peak changes in perfusion pressure are used to measure pressure changes from recorded data. The blood flow is maintained on a constant level. Therefore, changes in pressure must reflect changes in resistance. Pressure is recorded with a polygraph using an Statham pressure transducer (P23AA). In addition to recording perfusion pressure, venous pressure is measured at two additional points along the vein. In order to

record venous pressure centrally from the site of perfusion, the shaft of a 27-gauge needle is placed into the end of a 10 cm piece of a thin Silastic tubing. At the other end, a 27 gauge needle is inserted and attached to a Statham pressure transducer (P23B). Pressure is recorded on a polygraph. One needle is inserted into the vein just proximal of the junction of the two veins. The second needle is placed into the vein so that the distance between the tip of the perfusion cannula and the first needle is the same as the distance between the two needles. In this way pressure decreases across the junction and an adjacent segment can be measured simultaneously. Injections of test compounds are made into the cannula between the pump and the saphenous vein. Changes in pressure measured by the three transducers are recorded. As standard, doses of 0.1–1.0 µg norepinephrine are injected.

EVALUATION

Responses to test drugs are measured in mm Hg and calculated as percentage of response to norepinephrine.

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A.8.3

Arterial Aneurysms

A.8.3.1

General Considerations

Abdominal aortic aneurysms represent a life-threatening condition characterized by chronic inflammation, destructive remodeling of the extracellular matrix and segmental dilatation of the aortic wall (Dobrin 1989; Ernst 1993). Several authors described animal models of aneurysms (Carrell et al. 1999; Dobrin 1999; Daugherty and Cassis 2004). Most studies were performed in mice. Abdominal aortic aneurysms are evoked by genetically defined defects in extracellular matrix maturation, increased degradation of elastin and collagen, aberrant cholesterol homeostasis, or enhanced production of angiotensin peptides.

The **Blotchy mouse** has an X-linked trait that leads to aortic aneurysms and subsequent fatal rupture in nearly all affected male mice (Brophy et al. 1988; Reilly et al. 1990).

Maki et al. (2002) showed that inactivation of the **lysyl oxidase gene Lox** leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice.

Aneurysm development has been noted in a number of mice with deficiencies in the components of the matrix metalloproteinase system (Silence et al. 2001, 2002).

The use of mice in atherosclerosis research was escalated by the development of mice that are deficient in either apoE or LDL receptors susceptible to aneurysm development (Ishibashi et al. 1994; Prescott et al. 1999).

Accelerated atherosclerosis, aortic aneurysms formation, and ischemic heart disease were found in apolipoprotein E and endothelial nitric oxide synthase double-knockout mice (Kuhlencordt et al. 2001). ACE inhibition reduces some symptoms of vascular pathology in apoE and eNOS compound-deficient mice (Knowles et al. 2000). Mice with deficiency of the LDL receptor-related protein (LRP) showed pathological changes in the aortic arch and abdominal aorta with substantial lengthening, dilatation, thickening and large aneurysms (Herz and Strickland 2001; Boucher et al. 2003).

Salt-sensitive aortic aneurysms and rupture in hypertensive transgenic mice that overproduce angiotensin II were described by Nishijo et al. (1998).

The infusion of elastase into the infrarenal segment has been used as model for abdominal aorta aneurysms in rats (Anidjar et al. 1990) and mice (Pyo et al. 2000).

Lee et al. (2001) described abdominal aortic aneurysms in mice lacking expression of inducible nitric oxide synthase.

Periarterial application of calcium chloride was used to induce aneurysms of the **rabbit** common carotid artery (Gertz et al. 1988) and in the rabbit aorta (Freestone et al. 1997).

This technique has been used in **mice** by Chiou et al. (2001) and Longo et al. (2002).

MODIFICATIONS OF THE METHOD

Nomoto et al. (2003) described the effects of two inhibitors of renin-angiotensin system on attenuation of postoperative remodeling after **left ventricular aneurysm** repair in rats.

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A.8.3.2

Angiotensin II-Induced Aortic Aneurysm in Mice

PURPOSE AND RATIONALE

Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice (Daugherty et al. 2000, 2001; Knowles et al. 2000; Wang et al. 2001; Manning et al. 2002, 2003; Deng et al. 2003; Martin-McNulty et al. 2003; Saraff et al. 2003).

Wang et al. (2005) reported that a Rho-kinase inhibitor attenuated angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-deficient mice by inhibiting apoptosis and proteolysis.

PROCEDURE

Animal Preparation

Osmotic minipumps (model 2004, Alzet) containing either PBS or Ang II (1.44 mg/kg per day) in PBS were implanted subcutaneously in 6-month-old apoE-KO male mice (Jackson Laboratories). Two days before saline and Ang II infusion, mice were provided with water (Ang II group) or water containing test compound at a concentration of 0.5 mg/ml (low-dose group) or 1.0 mg/ml (high-dose group). Age-matched apoE-KO mice without any treatment were used as naïve controls. At the end of the 30-day treatment period, mice were euthanized, and the hearts were perfused with DEPC in saline. The arterial tree was rapidly dissected from fat and connective tissue and snap-frozen in liquid nitrogen.

MORPHOLOGICAL EXAMINATION

Quantification of Aneurysm Formation

After the aorta was dissected free from the surrounding connective tissue, images were recorded with a digital camera and later used to measure the outer diameter of the suprarenal aorta at the midpoint between the diaphragm and right renal artery. A commonly used

clinic standard to diagnose abdominal aortic aneurysm is an increase in aortic diameter of $\approx 50\%$ (Johnston et al. 1991). The average diameter of the normal suprarenal aorta in naïve control mice is 0.8 mm. A threshold of 1.22 mm was set as evidence of an incidence of aneurysm formation. Aneurysm severity was assessed with a scoring system described by Daugherty et al. (2001): type 0, no aneurysm (the suprarenal region of the aorta was not obviously different from naïve apoE-KO mice without Ang II treatment); type I, a dilated lumen with no thrombus; type II, remodeled tissue often containing thrombus; type III, a pronounced bulbous form of type II containing thrombus; and type IV, multiple aneurysms containing thrombus. To analyze this measurement semiquantitatively, the numerical score assigned to the type of aneurysm for each animal in a group was averaged to generate a pathology score for statistical comparison.

Quantification of Atherosclerotic Lesion Area

The left and right carotid arteries and the aortic arch were dissected, excised, opened longitudinally, and pinned down on wax-coated Petri dishes. Atherosclerotic lesions were visible without staining. Images of the open luminal surface of the vessels were captured with a digital camera (Sony) mounted on a dissecting microscope. The atherosclerotic lesion area was quantified by use of the C-Simple system (Compix) and expressed as a percentage of the total luminal surface area (Wang et al. 2000, 2001; Martin-McNulty et al. 2003).

Histology and Immunohistochemical Staining

Two representative suprarenal aortas from each group were fixed in formalin, embedded in paraffin, and cut into 5- μm -thick sections. The adjacent sections were stained with hematoxylin and eosin or by the immunohistochemical method. To identify macrophages in the aortic wall, a purified rat anti-mouse Mac-3 monoclonal antibody (BD Pharmingen) was used for immunohistochemistry staining (PhenoPath Laboratories). To localize cells undergoing nuclear DNA fragmentation, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed using an *in situ* apoptosis detection kit (Roche Biochemicals) (Song et al. 2000; Feng et al. 2002). Paraffin sections were deparaffinized and rehydrated. Sections were then washed with PBS and incubated with proteinase K (20 $\mu\text{g}/\text{ml}$) for 20 min. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in methanol at room temperature. TdT, which catalyzes a template-independent addition of

deoxyribonucleotide to 3-OH ends of DNA, was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragment *in situ*. The TUNEL signal was then detected with an anti-digoxigenin antibody conjugated with peroxidase and developed with diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and cleared before coverslips were placed. Both positive and negative control slides were processed at the same time in each experiment. The presence of apoptotic cells was scored as nuclear staining, with a distinctive morphological appearance associated with cell shrinkage and chromatin condensation.

EVALUATION

Results are presented as mean \pm SE for the number of animals used. Statistical comparison for the incidence of abdominal aortic aneurysms was performed by χ^2 analysis. Multiple comparison of mean values was performed by ANOVA, followed by a subsequent Student-Newman-Keuls test for repeated measures. Differences were considered statistically significant at a value of $P < 0.05$.

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A.8.4

Angiogenesis and Anti-Angiogenesis

A.8.4.1

General Considerations

Regulation of new blood vessel formation, angiogenesis, is precisely programmed throughout the lifetime of vertebrates. Besides the role of angiogenesis in normal function, it is an essential component of disease processes, including tumor growth, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman and Klagsbrun 1987; Klagsbrun and D'Amore 1991; Folkman and Shing 1992). Multiple factors that stimulate angiogenesis either directly or indirectly have been described, including the fibroblast growth factor family (Esch et al. 1985), vascular endothelial growth factor (Leung et al. 1990; Thomas 1996; Ferrara and Davis-Smyth 1997), epidermal growth factor (Gospodarowicz et al. 1979), transforming growth factor- α and - β (Schreiber et al. 1986; Yang and Moses 1990), tumor necrosis factor- α (Leibovich et al. 1987), angiogenin (Fett et al. 1985), CYR61, a product of a growth factor-inducible immediate early gene (Babic et al. 1998) ect.

The pharmacological inhibition of angiogenesis is of considerable interest in the development of new therapeutic modalities for the treatment of diseases such as diabetic retinopathy, atherosclerosis, hemangiomas, rheumatoid arthritis and cancer, in which pathological angiogenesis occurs (Ezekowitz et al. 1992; Folkman and Shing 1992; Fan and Brem 1992; O'Brien et al. 1994). Several natural inhibitors of angiogenesis were described, such as thrombospondin (Good et al. 1990), somatostatin (Barrie et al. (1993), angiostatin, isolated from a subclone of Lewis lung carcinoma, (O'Reilly et al. 1994, 1996), endostatin, a 20 kDa angiogenesis inhibitor from a murine hemangioendothelioma which is a C-terminal fragment of collagen XVIII (O'Reilly et al. 1997; Dhanabal et al. 1999), vasostatin (Pike et al. 1998).

Angiogenesis was studied *ex vivo* by culturing rat or mouse aortic rings in collagen gel (Zhu et al. 2003). Unlike rat aorta explants, unstimulated mouse aortic rings were unable to spontaneously produce an angiogenic response under serum-free conditions. They, however, responded to basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), generating networks of branching neovessels.

Couffinhal et al. (1998) published a mouse model of angiogenesis. The femoral artery of one hind-limb was ligated and excised. Laser Doppler perfusion imaging was employed to document the consequent reduction in hind-limb blood-flow, which typically persisted for up to 7 days. Neovascularization was shown to develop in association with augmented expression of VEGF mRNA and protein from skeletal myocytes as well as endothelial cells from the ischemic hind-limb.

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A.8.4.2

Endothelial Cell Proliferation

PURPOSE AND RATIONALE

Human umbilical vein endothelial cells (HUVEC) were used by various authors to study endothelial cell proliferation (Bussolino et al. 1992; Benelli et al. 1995; Danesi et al. 1997; Hu 1998; Iurlaro et al. 1998; Vacca et al. 1999; Xin et al. 1999).

PROCEDURE

The HUV-EC-C human endothelial cells (American Type Culture Collection, Rockville, MD) are cultured at 37°C and 5% CO₂ in 90% Ham's F12K, 10% fetal bovine serum, 30 µg/ml endothelial cell growth factor, 100 µg/ml heparin, and 4 mM L-glutamine. The effect of test compound on HUV-EC-C cell proliferation is evaluated on 3 × 10³ cells/well in 24-well plates. After 24 h, the test compound in various concentrations of the vehicle are added, and plates are incubated for 72 h. Cells are then harvested with trypsin/EDTA and counted by an hemocytometer.

EVALUATION

Results are expressed as number of cells in vehicle and compound-treated cultures and are the mean of three separate experiments ± SE.

MODIFICATIONS OF THE METHOD

In addition to human umbilical vein endothelial cells, Pike et al. (1998) used fetal bovine heart endothelial cells and measured DNA synthesis by [³H]thymidine deoxyribose uptake.

Oikawa et al. (1991) used vascular cells from bovine carotid arteries and tested cell proliferation in a collagen gel and cell migration in a Boyden chamber.

Bovine capillary endothelial cells were used by Folkman et al. (1979), Clapp et al. (1993), O'Reilly et al. (1997), Cao et al. (1999).

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A.8.4.3

Chorioallantoic Membrane Assay

PURPOSE AND RATIONALE

The chick chorioallantoic membrane assay, originally described by Auerbach et al. (1974), has been used with some modifications by several authors to test angiogenesis and inhibition of angiogenesis, e.g., by Taylor and Folkman (1982), Crum et al. (1985), Vu et al. (1985), McNatt et al. (1992, 1999), Barrie et al. (1993), Clapp et al. (1993), Gagliardi and Collins (1993), Benelli et al. (1995), Ribatti et al. (1995), Klauber et al. (1996), Oikawa and Shimamura (1996), Danesi et al. (1997), O'Reilly et al. (1997), Iurlaro et al. (1998), Cao et al. (1999), Vacca et al. (1999).

PROCEDURE

Fertilized White Leghorn chicken eggs are incubated at 37°C at constant humidity. On incubation day 3, a square window is opened in the shell and 2 to 3 ml of albumen is removed to allow detachment of the developing chorioallantoic membrane (CAM). The window is sealed with a glass and the eggs are returned to the incubator. On day 8, 1 mm³ gelatin sponges loaded

with 3 μ l phosphate-buffered saline alone as the negative control or containing 3 μ g (1 mg/ml) of the angiogenic recombinant basic fibroblast growth factor alone as positive control, or together with various doses of test compound, are implanted on top of the CAM. The sponge traps the sample and allows slow release of the product. CAM are examined daily until day 12, when the angiogenic response peaks. On day 12, blood vessels entering the sponge within the focal plane of the CAM are recognized microscopically, counted by two observers in a double-blind fashion under a Zeiss SR stereomicroscope, and photographed *in ovo* with the MC63 Camera system (Zeiss, Oberkochen, Germany). To better highlight vessels, the CAM are injected into a large allantoic vein with India ink solution, fixed in Serra's fluid, dehydrated in graded ethanols, and rendered transparent in methylbenzoate. On day 12, after microscopic counting, the embryos and their membranes are fixed *in ovo* in Bouin's fluid. The sponges and the underlying and immediately adjacent CAM portions are removed, embedded in paraffin, sectioned at 8 μ m along a plane parallel to the CAM surface, and stained with a 0.5% aqueous solution of toluidine blue.

EVALUATION

Angiogenesis is measured by a planimetric point count method (Ribatti et al. 1999): four to six 250 \times magnification fields covering almost the whole of every third section within 30 serial slides of each sponge per sample are analyzed within a superimposed 144 intersection point square reticulum of 0.125 mm². Only transversely sectioned microvessels, ie, capillaries and venules with or without a 3 to 10 μ m lumen occupying the intersection points, are counted and calculated as the mean \pm 1 SD per section, per CAM, and groups of CAM. Statistical significance of differences is calculated by comparing the data from each experiment to their controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Oh et al. (1997) studied the lymphatics of differentiated avian chorioallantoic membrane using microinjection of Mercox resin, semi- and ultrathin sectioning, immunohistochemical detection of fibronectin and α -smooth muscle actin, and *in situ* hybridization with vascular endothelial growth factor VEGFR-2 and VEGFR-3 probes.

Using the chick chorioallantoic membrane assay, Giannopoulou et al. (2003) showed that amifostine, an inorganic thiophosphate-cytoprotective agent, inhibits angiogenesis *in vivo*.

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A.8.4.4

Cornea Neovascularization

PURPOSE AND RATIONALE

Neovascularization of the rabbit cornea has been used by several authors to study inhibition of angiogenesis (Gimbrone et al. 1974; Crum et al. 1985; BenEzra et al. 1987, 1997; Klauber et al. 1996; McNatt et al. 1999; Jousseaume et al. 1999).

PROCEDURE

New Zealand White rabbits are anesthetized for surgery and quantification of newly developed blood vessels with 5 mg/kg xylazine hydrochloride and 35 mg/kg ketamine hydrochloride *i.m.* Corneal blood vessels are induced by basic fibroblast growth factor which is applied in carrier pellets. These pellets are produced by dispersing 50 µl of 2% methylcellulose containing 500 ng human recombinant basic fibroblast growth factor diluted in 10 µl phosphate-buffered saline in plastic rods with a diameter of 4 mm. Dried pellets are folded twice and implanted intrastromally in the 12 o'clock position into a corneal tunnel. This tunnel is created by a central cut of approximately 50% depth and extended into the peripheral cornea to a point 2.0 mm away from the limbus. Following implantation, the central entrance of the tunnel is closed with a single 10–0 nylon suture in order to ensure that the tear film does not dissolve the pellet and uncontrolled liberation of the growth factor is prevented.

The test substance is dissolved in a viscous gel containing 0.002% polyacrylic acid, 0.04% sorbitol, and 0.001% cetrimide in a watery base. The eyes are treated once daily with 0.1 ml of this gel which is applied in the lower conjunctiva sac. The eyes are closed for several seconds to avoid loss of the substance. Each animal's contralateral eye receives gel without test substance and serves as control. Control animals receive the viscous gel without test substance.

Animals are observed daily under an operating microscope, and vascular growth is documented on days 6, 9, 12, and 16 after surgery. The number of blood vessels, their length and the dimension of the

vascularized area are quantified with a caliper under the operating microscope as well as on standardized photographs. On every observation day corneas are stained with fluorescein in order to show epithelial irregularities due to the topical treatment.

EVALUATION

Differences between treated eyes and controls are tested for significance using unpaired Student's *t*-test.

MODIFICATIONS OF THE METHOD

Damms et al. (1997) characterized the neovascularization that follows the intracorneal injection of bovine albumin in rabbits as a model of angiogenesis. New Zealand white rabbits received intracorneal injections of phosphate buffered saline with and without various amounts of bovine albumin. The rabbits were co-sensitized or pre-sensitized by intramuscular bovine albumin. The corneal response was quantified by ranking photographs taken periodically after the injection.

Babic et al. (1998) tested an angiogenesis promotor in the corneal pocket angiogenesis assay in **rats**.

Xin et al. (1999) studied inhibitors of angiogenesis in the corneal angiogenesis assay in rats. A 1.5 mm incision was made approximately 1 mm from the center of the cornea in anesthetized Sprague Dawley rats. Using a curved spatula, the incision was bluntly dissected through the stroma toward the outer canthus of the eye. A hydron pellet (2×20 mm) containing 200 ng vascular endothelial growth factor and 100 ng sucralfrate was inserted into the base of the pocket.

Foschi et al. (1994), Benelli et al. (1995), Danesi et al. (1997) studied neovascularization of rat cornea induced by **chemical injury**. Both eyes of ether-anesthetized rats were cauterized by applying a AgNO₃/KNO₃ (1:1, w/w) applicator to the surface of the cornea eccentrically at a point approximately 2 mm from the corneoscleral limbus. Rats were treated 4 times daily for 6 days with eye drops. The eyes were examined by slit-lamp microscopy daily for 6 days to evaluate the growth of the vessels. On the 6th day after cauterization, the rats were anesthetized and the upper body perfused through a cannula inserted in the ascending aorta with Ringer's solution until the normal pink color of the fundi disappeared and then with a mixture of 10% India ink/6% gelatin in Ringer's solution. The eyes were enucleated and placed in 4% formaldehyde. The cornea and a 1 mm rim of adjacent scleral tissue were dissected from the rest of the globe

and three full thickness peripheral radial cuts were made to allow flattening of the cornea. The corneas were then placed on a glass slide in mounting media, magnified, and photographed. The area occupied by blood vessels was calculated and the area vascularization of drug treated animals was compared to that of control rats.

Kenyon et al. (1996), Cao et al. (1999) performed the corneal micropocket assay in **mice**.

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A.8.4.5**Rat Subcutaneous Air Sac Model****PURPOSE AND RATIONALE**

Lichtenberg et al. (1997, 1999) recommended the subcutaneous air sac model in rats as a simple method for *in vivo* screening of antiangiogenesis. Subcutaneous injection of air in the rat results in the formation of an air pouch. If air pouches are kept inflated by repeated injections of air they develop a structure of synovial lining (Edwards et al. 1981). The subcutaneous air pouch appears more like a bursal cavity than a synovial joint (Kowanko et al. 1986) and after 8–10 days the cells of the air sac appear as a transparent membrane on which the formation of new vessels can be studied.

PROCEDURE

Under anesthesia 10–15 ml of air is introduced dorsally to female Sprague Dawley rats weighing 150–180 g by subcutaneous injections using a 25 gauge needle to produce an air sac located approximately 4–5 cm behind the head of the animal. The air sacs are re-inflated every fourth day. The wall of the air sac becomes progressively thicker with time and after approximately 10 days a sufficient lining of cells has been established. For sponge implantation, the animals are anesthetized again. A 1.5 cm incision is made through the clipped skin covering the air sac and blunt dissection is used to open a 2 cm deep cavity towards the cranial base of the air sac by careful separation of the skin from the membrane. A cellulose implant (Spontex sponge) with a diameter of 8 mm is carefully pressed into the cavity of the membrane away from the incision site and the incision closed by sutures. The animals are treated for 10 days with various doses of test compound in a volume of 10 ml/kg or vehicle. The subcutaneous injection is made under light CO₂/O₂ anesthesia into the hind leg 5–7 cm away from the air sac. This injection site is chosen to eliminate any risk of inducing irritative side-effects on the membrane. After 10 days treatment, the animals are sacrificed after having received 20 min before an injection of 1 µCi of ¹²⁵I-labelled immunoglobulin via the tail vein. The overlying skin of the air sac is removed to expose the transparent membrane. The extent of vascular proliferation is scored *in situ*:

1+: slight background vascularization;
 2+: few new vessels reach the sponge;
 3+: many new vessels reach and penetrate the implant;
 4+: very intense formation of new vessels which reach and penetrate the implant.

Following *in situ* scoring, the implant and the membrane from each animal are placed in the same plastic vial containing 10% formalin and the radioactivity is measured in a γ -counter. The implants are examined microscopically after staining with haematoxylin and eosin.

EVALUATION

The extent of vascular proliferation scored *in situ* is compared between vehicle and treated animals by the Wilcoxon test. The angiogenic response measured by ¹²⁵I-activity in cpm is subjected to analysis of variance followed by Dunnett's *t*-test to compare each dose with the vehicle. The cpm's are log-transformed to obtain variance homogeneity. The correlation between *in situ* scores and cpm is estimated by Spearman's rank correlation coefficient after ranking cpm values.

MODIFICATIONS OF THE METHOD

In a further study, Lichtenberg et al. (1999) inoculated vascular endothelial growth factor producing tumor cells subcutaneously directly on the membrane, and the formation of vessels was measured 8 days later. Furthermore, slow-release pellets containing angiogenic factors, basic fibroblast growth factor or vascular endothelial growth factor, were implanted on the subcutaneous membrane.

Nakamura et al. (1999) studied suppression of angiogenesis induced by S-180 mouse tumor cells in the dorsal air sac assay in **mice**.

Funahashi et al. (1999) developed a mouse dorsal air sac model for quantifying *in vivo* tumor-induced angiogenesis which is determined by measuring the blood volume in an area of skin held in contact with a tumor cell-containing chamber, using ⁵¹Cr-labeled red blood cells.

Schreiber et al. (1986) described the **hamster cheek pouch assay** for testing angiogenic/antiangiogenic activity.

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A.8.4.6

Mesenteric Window Angiogenesis Model

PURPOSE AND RATIONALE

Norrby et al. (1986, 1990, 1995) described the mesenteric window assay in rats for quantitative measurement of induction and inhibition of angiogenesis. The tissue being used is the membranous, ‘window’-like parts of the mesentery which is normally vascularized and appears to lack significant physiologic angiogenesis. Since the mesenteric window natively measures only 5–10 μm in thickness, the vasculature is virtually two-dimensional. Due to the structural and metabolic simplicity of the test tissue, the mesenteric window microvasculature is regarded as an ideal test system for establishing the functional influences of defined factors (Zweifach 1973).

PROCEDURE

Angiogenesis is induced by i.p. injection of the mast-cell secretagogue compound 48/80 twice daily for 4.5 days to male Sprague Dawley rats weighing about 225 g. Test compounds or saline are injected s.c. 1 h before each injection of compound 48/80.

Angiogenesis is quantified by microscopically counting the number of vessel profiles per unit length of the mesenteric window in 4 microtome sections per specimen, cut perpendicularly to the surface, from the central part of the window. This reflects the degree of branching, the degree of tortuosity and the degree of spatial expansion of the vasculature. Four specimens per animal are analyzed.

Four mesenteric window specimens are spread, fixed on objective slides and stained with toluidine blue to measure the relative vascularized area. Three randomly selected vascular view fields per mesenteric-window spread are analyzed for microvascular length per unit area of vascularized tissue. The total microvascular length is computed from the vascularized area of each animal multiplied by the mean microvascular length for the corresponding treatment group.

EVALUATION

The non-parametric two-tailed Mann-Whitney U rank sum test for unpaired observations is used for statistical analysis.

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A.8.4.7

Quantification of Vascular Endothelial Growth Factor-C

PURPOSE AND RATIONALE

Vascular endothelial growth factor (VEGF) has achieved considerable therapeutic interest (Claus 1998; Enholm et al. 1998; Ferrara et al. 2004).

Weich et al. (2004) described an ELISA for quantification of vascular endothelial growth factor-C.

PROCEDURE

Materials and Methods

The fully processed rat protein dNdc-VEGF-C and dNdc-VEGF-D were produced in insect cells and purified from supernatants (Kirkin et al. 2001). Human dNdc-VEGF-C and soluble VEGFR-3 production was achieved according to (Joukov et al. 1997; Hornig et al. 1999). A polyclonal antibody against rat VEGF-C was developed in rabbits (antibody 4080; BioGenes Berlin). A total amount of 1.2 mg rat VEGF-C (containing a C-terminal 6His-tag) was used for immunization of two New Zealand white rabbits. After immunization with 0.1 mg protein, each rabbit was boosted on days 7, 14, 28, 56 and 84 with the same amount. The dilution of the serum for half-maximal titer was 1:10,000. Total IgG from rabbit serum was isolated using HiTrap Protein A Sepharose columns (Amersham Bioscience, Freiburg).

Generations of Cell Lines, Serum-Free Cell Culture Supernatants, Lysates and Tissue Sample Preparation

Experiments were conducted using three human (PC-3 cells, 293 cells, COLO 800 cells) and four rat (10AS, ARIP, BRL3A, MT-450) tumour cell lines and 1640 medium (Gibco-BRL, Bethesda, Md., USA). Stable transfected clones were selected and tested for VEGF-

C expression and secretion using a polyclonal anti-VEGF-C antiserum.

The cells were grown to 80% confluence in 75-cm² tissue culture flasks (Nunc, Roskilde, Denmark). Conditioned media was collected under low-serum (2%) growth conditions. Tumor tissues were snap-frozen in liquid nitrogen and homogenized in RIPA buffer (0.1% SDS, 1% IGEPAL CA-630, 0.5% Na-deoxycholate, protease-inhibitor cocktail in phosphate-buffered saline).

VEGF-C Sandwich-ELISA

The VEGF-C antibody 4080 was isolated from serum using HiTrap Protein-A Sepharose columns. Then, depletion of the anti-his antibody fraction was done by antigen-affinity purification using 10 mg of immobilized 6H-tagged TxnTb protein [Tryparedoxin (Txn) from *Trypanosoma brucei* (Tb)]. Antigen-affinity purification for the antibody 4080 was performed by immobilizing 1 mg rat VEGF-C on an NHS-activated HiTrap column (Amersham Bioscience, Freiburg). The development of a highly sensitive and specific sandwich ELISA for VEGF-C was done using standard methods. Rabbit IgG 4080 (10 µg/ml) was used for coating and the antigen-affinity purified and biotinylated antibody 4080 at 1 µg/ml was used as a detector antibody. Biotinylation of antibody 4080 was done with 6 mg IgG in 100 mM carbonate buffer, pH 8.5 at 3 mg/ml with using biotin-amidohexanoic acid NHS (Sigma, St. Louis, Mo., USA). The molecular ratio biotin:protein was 30:1. As a standard, human and rat dNdC-VEGF-C was used over a concentration range between 0.1 and 6.25 ng/ml. For visualization of the detector, streptavidin-enzyme conjugate was used (Endogen, Woburn, Mass., USA) followed by the addition of TMB (tetra-methyl-benzidine; Roche Mannheim, Germany).

EVALUATION

After stopping the reaction with 1 M H₂SO₄, the absorbance was measured at 450 and 620 nm with an ELISA plate reader (Labsystems, Finland). Generally, the samples were analyzed in different dilutions, measuring each dilution in duplicate. Samples were diluted at least 1:2 with sample diluent (BenderMedSystems, Vienna).

MODIFICATIONS OF THE METHOD

Rissanen et al. (2003) found that VEGF-D is the strongest angiogenesis and lymphangiogenic factor among VEGFs delivered into skeletal muscle via adenoviruses.

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A.8.4.8

Inhibitors of Vascular Endothelial Growth Factor

PURPOSE AND RATIONALE

Inhibitors of vascular endothelial growth factor have gained therapeutic interest (Whittles et al. 2002; Gingrich et al. 2003; Hamma-Kourbali et al. 2003; Verheul and Pinedo 2003;

Fernandez et al. 2004; Roberts et al. 2004; Baka et al. 2006). In particular, VEGF Trap, a soluble decoy receptor comprising portions of VEGF receptors 1 and 2, has been studied (Wulff et al. 2001; Holash et al. 2002; Kim et al. 2002; Hood and Cheresch 2003; Saishin et al. 2003; Fukasawa and Korc 2004; Fraser et al. 2005; Hu et al. 2005; Lau et al. 2005).

Byrne et al. (2003) found that vascular endothelial growth factor-Trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model.

PROCEDURE

Cell Lines

Ascites fluid from athymic mice previously inoculated with OVCAR-3 cells was used. Cells from the SKOV-3 human cystadenocarcinoma cell line were grown in McCoy's 5a medium with 1.5 mM L-glutamine, penicillin, and streptomycin, supplemented with 10% FCS. Cells were grown to confluence and harvested by trypsinization with 0.25 mg/ml trypsin/EDTA and suspended in PBS before inoculation into mice.

Animals

Athymic Balb c *nu/nu* mice were housed under pathogen-free conditions and fed autoclaved pellets and water.

Retroviral Constructs

The pLZR Phoenix vector was modified by the addition of a MCS followed by an internal ribosome entry sequence-GFP cassette (Rommel et al. 1999). Thus, genes subcloned into the MCS produce bicistronic constructs under the control of the viral 5' long terminal repeat. The entire coding sequence of mVEGF₁₆₄ was inserted into the MCS for the construct used to transduce cells with VEGF, whereas the MCS was left empty for the GFP-only vector. Constructs were transfected into Amphotrophic packaging lines to produce infective virus using standard techniques (Grignani et al. 1998).

SKOV-3 MODEL

SKOV-3 cell lines were infected with Amphotrophic viruses encoding either mVEGF₁₆₄ and GFP or GFP only. Cells that were successfully transduced with the retroviruses were collected by FACS using a Cytomation MoFlo (Fort Collins, Colo., USA) with fluorescence emission from GFP measured with a 530/540 nm bandpass filter. More than 50% of the cells were GFP positive after infection, allowing $>4.0 \times 10^5$ cells to be collected and used to establish cell lines. To verify viral transduction, cells were resorted several days later and found to be $>80\%$ positive for GFP expression. Cells were then expanded, aliquoted, and frozen. All experiments were performed with an aliquot expanded by four to five passages and tested for viability before injection.

In Vivo Adenoviral and SKOV-3 Studies

Adenoviral constructs were achieved according to Thurston et al. (2000). Adenoviral plaque-forming units (5.0×10^8) or 1.0×10^7 SKOV-3 cells were suspended in a volume of 300–400 μ l of PBS or serum-

free cell culture medium and injected i.p. into female nude mice. VEGF-Trap or control buffer was delivered twice weekly at 25 mg/kg via s.c. injection in a volume of 50–200 μ l. Mice were assessed daily for general health and development of ascites and weighed at least twice weekly. Animals were sacrificed if they had lost $>10\%$ of body weight or had persistent ascites on three consecutive assessments. After sacrifice, ascites was removed with a sterile thin caliber plastic transfer pipette and quantified, and hematocrit was measured.

OVCAR-3 MODEL

OVCAR-3 cells obtained from ascites fluid were prepared (Hu et al. 2002). Briefly, 2×10^6 cells in 500 μ l of RPMI 1640 were injected i.p. into athymic Balb/C nude (*nu/nu*) mice. Fourteen days after inoculation, blinded administration of VEGF-Trap or human Fc as control was initiated at a dose of 25 mg/kg. Injections were given s.c. in the nape of the neck using a 28.5-gauge needle and a 0.5-ml insulin syringe. Injections (0.05 ml) were administered twice weekly throughout the experimental period. Body weight and abdominal circumference were quantified twice weekly. In addition, animals were monitored daily for evidence of advanced disease (listlessness, extensive swelling of the abdominal cavity). At the end of the experiment, all remaining mice underwent euthanasia with CO₂ followed by cervical dislocation. The volume of ascites was measured, and tumors were excised and weighed. Immediately before sacrifice, mice received i.v. injection with FITC lycopersicon lectin (see below).

Tumor Vasculature

According to Holash et al. (2002) s.c. tumors were established. After small s.c. tumors became palpable (1 week after implantation), treatment with the VEGF-Trap was initiated. VEGF-Trap or an equivalent volume of vehicle was delivered twice weekly s.c. at the nape of the neck. Tumor vasculature was visualized by using antibodies to platelet-endothelial cell adhesion molecule for immunohistochemistry.

VEGF-Trap-treated OVCAR-3 tumor-bearing mice and control, untreated tumor-bearing mice were anesthetized by i.m. injection with ketamine (87 mg/kg) and xylazine (13 mg/kg), followed by i.v. injection with 100 μ l of FITC lycopersicon lectin or 100 μ l of Cy3 albumin (Jackson Immunology Research, West Grove, Pa., USA). Then 10 min later, mice were perfused through the ascending aorta with 4% paraformaldehyde in PBS for 2 min. Tumors and control organs were extracted and placed in fixative for 1–2 h followed by immersion in 30% sucrose/PBS

overnight, embedded in OCT, cryostat sectioned, and viewed by fluorescence microscopy.

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Chapter B

Pharmacological Assays in Thrombosis and Haemostasis¹

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¹Contributed by Shaker A. Mousa based on contributions by M. Just and V. Laux.

B.8 Safety Assays in Thrombosis and Haemostasis 455

B.1 General Introduction

Cardiovascular, cerebrovascular, and venous thromboembolic disorders continue to be the leading causes of death throughout the world. Over the past two decades, great advances have been made in the pharmacological treatment and prevention of arterial and venous thrombotic disorders (e. g., tissue plasminogen activators, platelet GPIIb/IIIa antagonists, and ADP receptor antagonists such as clopidogrel, low-molecular weight heparins, and direct thrombin inhibitors). New research is leading to the next generation of antithrombotic compounds such as direct coagulation FVIIa inhibitors, tissue factor pathway inhibitors, gene therapy, and orally active direct thrombin inhibitors and coagulation factor Xa (FXa) inhibitors. In vitro assays as well as animal models of thrombosis have played and will continue to play crucial roles in the discovery and validation of novel drug targets, the selection of new agents for clinical evaluation, and the provision of dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. This comprehensive manual presents the pivotal models that led to the development of drugs that have proven to be effective clinically. The major issues regarding the use of animal models of thrombosis—such as the use of positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species-specificity, and pharmacokinetics—are highlighted. Finally, the use of genetic models in thrombosis/hemostasis research and pharmacology is also presented.

B.2 In Vitro Tests

B.2.1 Blood Coagulation Tests

PURPOSE AND RATIONALE

The coagulation cascade consists of a complex network of interactions resulting in thrombin-mediated conversion of fibrinogen to fibrin, which is one major component of a thrombus. The coagulation cascade can be initiated either by the “exogenous pathway,” the release of thromboplastin (tissue factor) leading to activation of factor VII to the tissue factor/factor VIIa

complex, or by the “endogenous pathway,” so-called contact activation leading via factors XII, XI and IX to the assembly of the tenase complex consisting of activated factors VIII and IX and Ca^{2+} on a phospholipid surface. Both complexes can activate factor X, which induces the formation of the prothrombinase complex consisting of factor X_a, factor Va and Ca^{2+} on a phospholipid surface. The latter leads to the activation of thrombin, which, in turn, cleaves fibrinogen to fibrin. The three coagulation tests (prothrombin time [PT], activated partial thromboplastin time [APTT], and thrombin time [TT]) allow one to differentiate between effects on the exogenous or endogenous pathway or on fibrin formation. The influence of compounds on the plasmatic blood coagulation is determined by measuring the coagulation parameters PT, APTT, and TT *ex vivo*.

PROCEDURE

Male Sprague-Dawley rats weighing 200–220 g receive the test compound or the vehicle (controls) by oral, intraperitoneal, intravenous, or other route of administration. After the end of the absorption time, they are anesthetized by intravenous injection of 60 mg/kg sodium pentobarbital. The caudal caval vein is exposed by a midline incision or by cardiac puncture and 1.8 ml blood is collected into a plastic syringe containing 0.2 ml 100 mM citrate buffer pH 4.5 (Behring Werke, Marburg). The sample is immediately agitated and centrifuged in a plastic tube at $1500 \times g$ for 10 min. Plasma is transferred to another plastic tube and the coagulation tests for the determination of TT, PT, and APTT are performed within 3 h.

In general, citrated plasma is coagulated by the addition of the respective compounds (see below), and the time to clot formation is determined in the coagulometer (= coagulation time).

For detailed laboratory diagnosis of bleeding disorders and assessment of blood coagulation see Palmer (1984) and Nilsson (1987).

Prothrombin Time (PT). An aliquot of 0.1 ml of citrated plasma is incubated for 1 min at 37°C. Then 0.2 ml of human thromboplastin (Thromborel, Behring Werke, Marburg) is added and the coagulometer (Schnittger+Gross coagulometer, Amelung, Brake) is started. The time to clot formation is determined. The PT measures effects on the exogenous pathway of coagulation.

Activated Partial Thromboplastin Time (APTT). To 0.1 ml of citrated plasma 0.1 ml of human placenta lipid extract (Pathrombin, Behring Werke, Marburg) is added and the mixture is incubated for 2 min at 37°C.

The coagulation process is initiated by the addition of 0.1 ml 25 mM calcium chloride when the coagulometer is started and the time to clot formation is determined. The APTT measures effects on the endogenous pathway of coagulation.

Thrombin Time (TT). To 0.1 ml of citrated plasma 0.1 ml of diethylbarbiturate-citrate buffer, pH 7.6 (Behring Werke Marburg) is added and the mixture is incubated for 1 min at 37°C. Then 0.1 ml of bovine test-thrombin (30 IU/ml, Behring Werke Marburg) is added and the coagulometer is started. The time to clot formation is determined. The TT measures effects on fibrin formation.

EVALUATION

Mean values of TT, PT, and PTT are calculated in dosage groups and vehicle controls. Statistical evaluation is performed by means of the unpaired Student's *t*-test.

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B.2.2

Thrombelastography

PURPOSE AND RATIONALE

Thrombelastography (TEG) was developed first by Hartert (1948). The thrombelastograph (Haemoscope Corp, Skokie, Illinois, USA) is a device that provides a continuous recording of the process of blood coagulation and subsequent clot retraction. The blood samples are transferred to cuvettes and maintained at 37°C. The cuvettes are set in motion around their vertical axes. Originally, a torsion-wire suspended mirror in the plasma remains immobile as long as the plasma is fluid. The cuvette and the mirror become dynamically related as fibrin forms, resulting in transmission of cuvette motion to the mirror. The mirror then oscillates with an amplitude governed by the specific mechanical properties of the clot and reflects its light to a thermopaper. The modern thrombelastograph transfers the analogous recording to a digital signal that is evaluated by a computer program.

PROCEDURE

TEG can be performed in either whole blood or in citrated platelet-rich or platelet-poor plasma after recalcification. Blood samples are obtained from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.7–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the compound by intravenous (i.v.), subcutaneous (s.c.), or oral administration. Ten or 20 min post dosing (i.v., s.c. administration) or 60, 90 or 180 min post dosing (oral administration) blood is collected. The blood samples are mixed with 3.8% trisodium citrate solution (1 part citrate solution to 9 parts blood) as anticoagulant. The citrated whole blood is recalcified by adding 0.4 ml isotonic calcium chloride solution. An aliquot of 0.36 ml of the recalcified whole blood is transferred to the prewarmed cup of the thrombelastograph. After the apparatus has been correctly adjusted and the samples sealed with liquid paraffin to prevent drying, the time for the whole procedure is noted. The thrombelastogram is recorded for 2 h.

EVALUATION

The following measurements are the standard variables of TEG:

1. Reaction time (*r*): the time from sample placement in the cup until onset of clotting (defined as amplitude of 1 mm). This represents the rate of initial fibrin formation.
2. Clot formation time (*k*): the difference from the 1 mm *r* to 20 mm amplitude. *k* represents the time taken for a fixed degree of viscoelasticity achieved by the forming clot, caused by fibrin build up and cross linking.
3. Alpha angle (α°): angle formed by the slope of the TEG tracing from the *r* to *k* value. It denotes speed at which solid clot forms.
4. Maximum amplitude (*MA*): greatest amplitude on the TEG trace. *MA* represents the absolute strength of the fibrin clot and is a direct function of the maximum dynamic strength of fibrin and platelets.
5. Clot strength (*G* in dynes per square centimeter): defined by $G = (5000 MA) / (96 - sMA)$. In a tissue factor-modified TEG (Khurana et al. 1997), clot strength is clearly a function of platelet concentration.
6. Lysis 30, Lysis 60 (*Ly30*, *Ly60*): Reduction of amplitude relative to maximum amplitude at 30 and 60 min after time of maximum amplitude. These parameters represent the influence of clot retraction and fibrinolysis.

| TEG Parameters | TF (25 ng) | LPS (0.63 ug) | Xa (0.25 nM) | | Thrombin (0.3 mU) |
|----------------|------------|---------------|--------------|--|-------------------|
| | | | Mean ± SEM | | |
| R (minutes) | 29.7 ± 2.3 | 23.4 ± 1.4 | 15.6 ± 2.9 | | 3.4 ± 0.6 |
| K (minutes) | 5.8 ± 1.0 | 7.6 ± 0.9 | 4.8 ± 0.5 | | 5.5 ± 0.8 |
| α (angle) | 45.0 ± 2.6 | 47.8 ± 3.2 | 61.5 ± 2.1 | | 57.8 ± 2.9 |
| MA (mm) | 58.2 ± 1.7 | 50.0 ± 2.0 | 65.0 ± 0.8 | | 50.1 ± 2.4 |

Table 1 Effect of various stimulus on platelet/fibrin clot dynamics as shown by Mousa et al. (2000)

Citrated human whole blood plus 2 mM calcium. Data represent mean for $n = 6 \pm \text{SEM}$.

MODIFICATIONS OF THE METHOD

Bhargava et al. (1980) compared the anticoagulant effect of a new potent heparin preparation with a commercially available heparin by TEG *in vitro* using citrated dog and human blood. Barabas et al. (1993) used fibrin plate assay and TEG to assess the antifibrinolytic effects of synthetic thrombin inhibitors. Scherer et al. (1995) described an endotoxin-induced rabbit model of hyper-coagulability for the study of the coagulation cascade and the therapeutic effects of coagulation inhibitors using various parameters, including TEG.

Khurana et al. (1997) introduced tissue factor-modified TEG to study platelet glycoprotein IIb/IIIa function and to establish a quantitative assay of platelet function. With this modification, Mousa et al. (2000) found two classes of glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists, one with high binding affinity for resting and activated platelets and slow platelet dissociation rates (class I) demonstrating potent inhibition of platelet function, in contrast to those with fast platelet dissociation rates (class II). Additionally, Mousa et al. (2005) utilized the TEG in phase II clinical trial in monitoring the efficacy of oral platelet GPIIb/IIIa antagonist on platelet/fibrin clot dynamics.

CRITICAL ASSESSMENT OF THE METHOD

Zuckerman et al. (1981) compared TEG with other common coagulation tests (fibrinogen, prothrombin time, activated thromboplastin time, platelet count, and fibrin split products) and found that there is a strong relationship between the thrombelastographic variables and these common laboratory tests. Mousa et al. (2005) and others expanded the use of TEG in differentiating among different antiplatelets, anticoagulants, and optimal combinations of both. Moreover, TEG has an increased sensitivity for detecting blood clotting anomalies; it contains additional information on the hemostatic process. This is due to the following: (1) the fact that most laboratory measurements end with the formation of the first fibrin strands while TEG measures the coagulation process on whole blood

from initiation of clotting to the final stages of clot lysis and retraction, and (2) the ability of TEG to use whole non-anticoagulated blood without influence of citrate or other anticoagulants.

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B.2.3

Chandler Loop

PURPOSE AND RATIONALE

The Chandler loop technique allows the production of *in vitro* thrombi in a moving column of blood (Chandler 1958). The thrombi generated in the Chandler device show morphology very similar to that of human thrombi formed *in vivo* (Robbie et al. 1997), with platelet-rich upstream sections (“white heads”) that are

relatively resistant to t-PA-mediated thrombolysis in contrast to the red blood-cell-rich downstream parts ("red tails") (Stringer et al. 1994).

PROCEDURE

One millimeter of non-anticoagulated whole blood is drawn directly into a polyvinyl tube with a length of 25 cm and an internal diameter of 0.375 cm (1 mm = 9.9 cm tubing). The two ends of the tube are then brought together and closed by an outside plastic collar. The circular tube is placed and centered on a turntable, tilted to an angle of 23°, and rotated at 17 rpm. At the moment the developing thrombus inside the tube becomes large enough to occlude the lumen, the blood column becomes static and moves around in the direction of rotation of the tube.

EVALUATION

Time to occlusion of the tube by the thrombus establishes a definite end point in this system.

MODIFICATIONS OF THE METHOD

Stringer et al. (1994) used this method to determine the influence of an anti-PAI-1 antibody (CLB-2C8) on the t-PA-induced lysis of Chandler thrombi *in vitro*. They used citrated blood and supplemented it with 5.8 μM [¹²⁵I]-labeled fibrinogen prior to recalcification. After generation in the Chandler loop, the thrombi were washed with isotonic saline and then cut transversally into an upstream (head) and a downstream part (tail). The radioactivity of both parts was determined in a gamma counter (pre-value). The head and the tail were then subjected to thrombolysis by adding 300 μl phosphate-buffered saline containing plasminogen (2 μM) and t-PA (0.9 nM). During the observation time of 240 min, aliquots of 10 μl were taken at 30, 60, 120, 180 and 240 min, and the radioactivity was determined. The relation of the measured radioactivity to the pre-value was expressed as percentage of clot lysis.

Van Giezen et al. (1998) used this method to differentiate the effect of an anti-PAI-1 polyclonal antibody (PRAP-1) on human or rat thrombi.

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B.2.4

Platelet Aggregation and Deaggregation in Platelet-Rich Plasma or Washed Platelets (Born Method)

PURPOSE AND RATIONALE

Platelets play a crucial role in primary hemostasis by forming hemostatic plugs at sites of vascular injury. Moreover, they contribute to intravascular thrombus formation mostly upon rupture of an atherosclerotic plaque. The contact of unactivated platelets to exposed subendothelial tissue leads to adhesion via two main mechanisms: binding of subendothelial von Willebrand factor (vWF) to the platelet GPIb-IX-V-complex at high shear rates and binding of collagen to two receptors, integrin $\alpha 2\beta 1$ and GPVI. Platelet adhesion initiates the reactions of shape change, secretion, and activation of GPIIb-IIIa-ligand binding sites. These reactions result in the formation of platelet aggregates. Activation of GPIIb-IIIa is also achieved through signaling by a number of agonists that bind to G-protein-coupled receptors. Consequently, for the measurement of platelet aggregation, platelets are activated by the addition of one of the following agonists to platelet-rich plasma (PRP) or washed platelets: ADP, arachidonic acid (forming thromboxane A₂) or U 46619, collagen, thrombin or TRAP, serotonin, epinephrine, PAF. The formation of platelet aggregates with stirring leads to changes in optical density that are monitored photometrically, usually for 4 min. The test has been developed originally by Born (1962a, and 1962b) and is used to evaluate quantitatively the effect of compounds on induced platelet aggregation *in vitro* or *ex vivo*. For *in vitro* studies, human PRP is preferred.

PROCEDURE

The test is carried out either *ex vivo* or *in vitro*. There are other commercial sources for the various agonists listed.

Table 2 Materials and solutions

| Anticoagulating substances | |
|-----------------------------------------------------------------------|-----------------|
| Hirudin (Sigma) or PPAK | 200 µg/ml |
| Trisodium citrate | 0.11 M |
| ACD solution | |
| Citric acid | 38 mM |
| Sodium citrate | 75 mM |
| Glucose | 124 mM |
| Platelet-aggregating substances (final concentrations in the test) | |
| ADP: for reversible or biphasic aggregation | 0.1–5 µM |
| ADP: for irreversible aggregation (Sigma) | 3–10 µM |
| Sodium arachidonate (Biodata) | 0.3–1 mM |
| Calcium ionophore A 23187 (Calbiochem) | 10 µM |
| Collagen (Hormonchemie) | 3 µg/ml |
| PAF-acether (C 16-PAF, Bachem) | 0.1 µM |
| Thrombin (Sigma) | 0.02–0.05 IU/ml |
| TRAP (SFLLRNP, Bachem) | 1–10 µM |
| U 46619 (ICN) | 1–10 µM |
| Ristocetin | 0.1–1 mg/ml |
| GPRP (fibrin antipolymerant, Bachem) | 0.5 mM |
| 4-channel aggregometer (PAP 4, Bio Data) | |

For *ex vivo* assays, mice, rats, or guinea pigs from either sex receive the test compound or the vehicle (for controls) by oral, intraperitoneal or intravenous administration. At the end of the absorption time, blood is collected by caval venipuncture under pentobarbital sodium anesthesia and xylazine (8 mg/kg i.m.) premedication.

From rabbits (Chinchilla strain, weighing 3 kg), blood is withdrawn by cardiopuncture under xylazine (20 mg/kg i.m.) sedation. The first blood sample (control) is collected before administration of the test compound, the second sample at the end of the absorption time of the test agent.

For *in vitro* assays, human blood is collected from the antecubital vein of adult volunteers who had not received any medication for the last two weeks.

PREPARATION OF PRP, PPP, AND WP

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Freshly collected venous blood is anticoagulated with hirudin (1 volume + 9 volumes of animal blood) or ACD solution (1 volume + 9 volumes of human blood) and centrifuged at 150 × g for 15 min to obtain platelet-

rich plasma (PRP). The PRP-supernatant is carefully removed, and the rest is further centrifuged at 1500 × g for 10 min to obtain platelet-poor plasma (PPP). PRP is diluted with PPP to a platelet count of 3 × 10⁸/ml before use in the aggregation assays. To obtain washed platelets (WP), 8.5 volumes of human blood are collected into 1.5 volumes of ACD and centrifuged as for PRP. PRP is acidified to a pH of 6.5 by addition of approximately 1 ml ACD to 10 ml PRP. Acidified PRP is centrifuged for 20 min at 430g. The pellet is re-suspended in the original volume with Tyrode's solution (mM: NaCl 120, KCl 2.6, NaHCO₃ 12, NaH₂PO₄ 0.39, HEPES 10, glucose 5.5; albumin 0.35%) and set to platelet count of 3 × 10⁸/ml.

For *ex vivo* assays, duplicate samples of 320 µl PRP from drug-treated and vehicle control subjects (for rabbits: control samples before drug administration) are inserted into the aggregometer at 37°C under continuous magnetic stirring at 1000 rpm. After the addition of 40 µl physiological saline and 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm.

For *in vitro* assays, 40 µl of the test solution are added to samples of 320 µl PRP or WP from untreated subjects. The samples are inserted into the aggregometer and incubated at 37°C for 2 min under continuous magnetic stirring at 1000 rpm. After the addition of 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm either for 4 min or until constant values for aggregation are achieved. In cases of thrombin activation of PRP, glycine-proline-aspartate-proline (GPRP) is added in order to avoid fibrin formation. In order to measure deaggregation, experimental compounds are added to stimulated PRP at 70 or 100% of control aggregation and monitoring is performed for further 10 min. Deaggregation is measured by the decrease of light transmission (see Haskell et al. 1989).

Studies should be completed within 3 hours after blood withdrawal.

EVALUATION

The transmission maximum serves as a scale for platelet aggregation (0% = transmission of PRP, 100% = transmission of PPP).

For *in vitro* assays:

1. Percent inhibition of platelet aggregation is determined in concentration groups relative to vehicle controls. Statistical significance is evaluated by means of the unpaired Student's t-test.
2. IC₅₀ values are determined from the non-linear curve fitting of concentration-effect relationships.

IC₅₀ is defined as the concentration of test drug for half maximal inhibition of aggregation.

3. Percent deaggregation is determined at 10 min after addition of compound; IC₅₀ is calculated from the concentration-effect relationship.

For *ex vivo* assays:

1. Mean values for aggregation in dosage groups are compared to the vehicle control groups (for rabbits: control values before drug administration). Statistical significance is evaluated by means of the Student's *t*-test (paired for rabbits; unpaired for others).
2. ED₅₀ values are determined from the dose-response curves. ED₅₀ is defined as the dose of drug leading to 50% inhibition of aggregation in the animals.

CRITICAL ASSESSMENT OF THE METHOD

The assay, introduced by Born (1962a and 1962b), has become a standard method in clinical diagnosis of platelet function disorders and of aspirin intake. Furthermore, the method is used in the discovery of antiplatelet drugs with the advantage of rapid measurement of a functional parameter in intact human platelets. However, processing of platelets during the preparation of PRP, washed or filtered platelets from whole blood results in platelet activation and separation of large platelets. Additionally, there is no standardization among the different laboratories due to variation in use of different tubes, different final agonist concentrations, and other technical differences.

MODIFICATIONS OF THE METHOD

Several authors have described modifications of the assay procedure. Breddin et al. (1975) described spontaneous aggregation of platelets from vascular patients in a rotating cuvette. Klose et al. (1975) measured platelet aggregation under laminar flow conditions using a thermostated cone-plate streaming chamber in which shear rates are continuously augmented and platelet aggregation is measured from light transmission through a transilluminating system. Marguerie et al. (1979, 1980) developed a method of measuring two phases of platelet aggregation after gel filtration of a platelet suspension (see below). Lumley and Humphrey (1981) described a method to measure platelet aggregation in whole blood (see below). Fratantoni and Poindexter (1990) performed aggregation measurements using a microtiter plate reader with specific modification of the agitation of samples. Comparison of the 96-well microtiterplate method with conventional aggregometry showed similar dose-response curves for thrombin, ADP, and arachidonic acid.

Ammit and O'Neil (1991) used a quantitative bioassay of platelet aggregation for rapid and selective measurement of platelet-activating factor. Mousa et al. (1994, 1998) and others utilized this assay for *in vitro* screening and *ex vivo* and antiplatelet efficacy in various species and in humans for platelet GPIIb/IIIa antagonists.

Yamanaka et al. (2005) performed platelet aggregations assays using an eight channel aggregometer (NBS HEMA TRACER 801, Nikobioscience, Tokyo, Japan) to test structure-activity relationships of potent GPIIb/IIIa antagonists.

Francischetti et al. (2000) used the Microplate Reader for studying a platelet aggregation inhibitor from the salivary gland of the blood-sucking bug, *Rhodnius prolixus*.

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B.2.5

Platelet Aggregation After Gel Filtration (Gel-Filtered Platelets, GFP)

PURPOSE AND RATIONALE

Triggering of platelet activation by low concentrations of ADP, epinephrine or serotonin—so-called weak platelet agonists—in plasma- and fibrinogen-free platelet suspensions does not result in platelet aggregation unless exogenous fibrinogen is added. As opposed to this, platelet aggregation induced by thrombin, collagen or prosta-glandin-endoperoxide—so-called strong agonists—is independent of exogenous fibrinogen because these substances lead to the secretion of intracellular platelet ADP and fibrinogen. Studies of platelet aggregation in gel-filtered platelets are performed in cases where the adhesive ligand fibrinogen or vWF is needed in a defined concentration or where plasma proteins could negatively interfere with the effect of compounds. The assay is mostly used to evaluate the influence of compounds on platelet GPIIb-IIIa or other integrins or on GPIb-IX-V. Mousa et al. (1994, 1998) and others have extensively utilized this assay to determine the impact of plasma protein binding on antiplatelet efficacy by comparing inhibition of platelet aggregation in GFP versus PRP.

PROCEDURE

Preparation of Gel-Filtered Platelets

The entire procedure is performed in plastic (polystyrene) tubes at room temperature according to Marquerie et al. (1979).

Blood is drawn from healthy adult volunteers who had no medication for the last two weeks. Venous blood (8.4 ml) is collected into 1.4 ml ACD-solution and centrifuged for 10 min at 120 g. The platelet-rich plasma (PRP) is carefully removed, the pH adjusted to 6.5 with ACD-solution, and centrifuged at 285 g for 20 min. The resulting pellet is resuspended in Tyrode's buffer (approx. 500 µl buffer/10 ml PRP). The platelet suspension is applied immediately to a Sepharose CL 2B column; equilibration and elution at 2 ml/min flow rate is done with Tyrode's buffer without hirudin and apyrase. Platelets are recovered in the void volume. Final platelet suspension is adjusted to 4×10^8 /ml. Gel filtered platelets (GFP) are kept at room temperature for 1 h until the test is started.

Table 3 Materials and solutions

| Acid-citrate-dextrose (ACD) solution | |
|----------------------------------------------------------------------------------|----------------|
| Citric acid | 0.8% |
| Sodium citrate | 2.2% |
| Glucose | 2.45% |
| Hirudin | 0.6 U/ml |
| Tyrode's solution | |
| NaCl | 137 mM |
| KCl | 2.7 mM |
| MgCl ₂ | 5.5 mM |
| NaH ₂ PO ₄ | 3.0 mM |
| HEPES | 3.5 mM |
| Glucose | 5.5 mM |
| Albumin | 0.2% |
| Hirudin | 0.06 U/ml |
| Apyrase | 40 µg/ml |
| pH | 7.2 |
| ADP | 10 µM |
| Thrombin | 0.02–0.05 U/ml |
| CaCl ₂ | 0.5 mM |
| Fibrinogen (American Diagnostica) | 1 mg/ml |
| von Willebrand factor | 10 µg/ml |
| Sepharose CL 2B (Pharmacia) | |
| Acrylic glass column (Reichert Chemietechnik, 3 cm inner diameter, 18 cm length) | |
| Aggregometer (PAP 4, Biodata) | |

Experimental Course

For the aggregation studies, GFP in Tyrode's buffer is incubated with CaCl₂ (final concentration 0.5 mM) with or without fibrinogen (final conc. 1 mg/ml) in polystyrene tubes. After 1 min, 20 µl of the test compound or the vehicle (controls) are added, and the samples are incubated for another 2 min. After the addition of 20 µl platelet agonist, changes in light transmission are recorded. The whole procedure is done under continuous magnetic stirring at 37°C (1000 rpm) in the aggregometer. Samples with added CaCl₂ but without fibrinogen identify proper exclusion of plasma proteins if neither spontaneous aggregation occurs nor aggregation in the presence of weak agonists. Full aggregatory response of GFP to 10 µM ADP shows intact platelets (with only minor pre-activation with gel filtration).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation. Each test compound is assayed with at least two different donor-GFPs; in the case of an anti-aggregating effect, the test is performed with 4–6 GFPs.

Mean values of the dosage groups are compared to the controls. Statistical significance is evaluated by means of the Student's *t*-test.

The percent inhibition of platelet aggregation in the dosage groups is calculated relative to the vehicle controls.

IC₅₀ values (50% inhibition of aggregation) are determined from the concentration-effect curves.

For detailed methodology and evaluation of different agents see Marguerie et al. (1979 and 1980), Markell et al. (1993), and Mousa et al. (1994 and 1998).

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B.2.6

Platelet Aggregation in Whole Blood

PURPOSE AND RATIONALE

The method uses a whole blood platelet counter that counts single platelets and does not require their separation from other blood cell types. Platelet aggregation is induced in anti-coagulated human whole blood samples by the addition of the aggregating agents arachidonic acid or collagen. The number of platelets is determined in drug-treated and vehicle control samples; the percentage of inhibition of aggregation and IC₅₀ values are calculated in dosage groups. The effect of compounds on other blood cells that secondarily can influence platelet aggregation is included in this test system. The method has been described by Lumley and Humphrey (1981) and by Cardinal and Flower (1980).

PROCEDURE

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Blood is drawn from healthy adult volunteers

Table 4 Materials and solutions

| | |
|--------------------------------------------------------------|------------------------|
| Anticoagulant: sodium citrate to induce platelet aggregation | 3.8% |
| Sodium arachidonate (Biodata) | 3.6×10^{-4} M |
| Collagen (Hormonchemie) | 10 µg/ml |
| Serono Hematology System 9000 or Sysmex Micrcellcounter F800 | |

who had not received medication for the last 2 weeks; 9 ml venous blood is anti-coagulated with 1 ml of sodium citrate and kept in a closed tube at room temperature for 30–60 min until the start of the test.

For the aggregation studies, 10 µl test substance or vehicle (control) is added to 480 µl citrated blood. Samples in closed tubes are pre-incubated for 5 min in a 37°C water shaker bath at 75 strokes/min. Then, 10 µl aggregating agent are added, and samples are incubated for another 10 min. The number of platelets (platelet count) is determined in 10 µl samples immediately before and 10 min after the addition of the aggregating agent ('initial platelet count', '10-min-platelet count' after adding platelet agonist ± antagonist) in a hematology cell counter.

The following samples for the determination of the platelet count are prepared in duplicate:

- control aggregation = spontaneous aggregation (without aggregating agent): 480 µl blood + 20 µl vehicle. Blood samples with >20% spontaneous aggregation are not used to test for induced aggregation.
- maximal aggregation: 480 µl blood + 10 µl vehicle + 10 µl aggregating agent. Values represent the maximal induced aggregation rate of the blood sample.
- test substance aggregation: 480 µl blood + 10 µl test substance + 10 µl aggregating agent.

EVALUATION

From the samples for maximal aggregation (vehicle), the percentage of maximal aggregation is calculated according to the following formula:

$$\begin{aligned} & \% \text{ maximal aggregation} \\ & = 100 - \frac{10\text{-min-platelet count} \times 100}{\text{initial plateletcount}} \end{aligned}$$

From the samples for test substance induced aggregation, the percentage of aggregation in dosage groups is

calculated according to the following formula:

$$\% \text{ aggregation} = \frac{10\text{-min-platelet count} \times 100}{\text{initial platelet count}}$$

IC₅₀ values (50% inhibition of aggregation) are determined from the dose-response curves (log concentration test substance versus % inhibition of aggregation).

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B.2.7

Platelet Micro- and Macro-Aggregation Using Laser Scattering

PURPOSE AND RATIONALE

- A new highly sensitive method to study platelet aggregation based on the measurement of mean radius or particle size makes it possible to record kinetics of formation of micro- and macro-aggregates in real time
- Sensitivity in measurements of spontaneous aggregation is higher than in routine light transmittance.

A new platelet aggregometer (AG-10; Kowa, Japan) that uses a laser-light-scattering beam has been introduced (Tohgi et al. 1996). Platelet aggregates, the size of which can be measured as total voltage of light-scatter intensity at 1.0-sec intervals for a 10-min period, can be divided into 3 ranges: small aggregates (diameter 9 to 25 μm), medium (26 to 50 μm), and large (>50 μm). Using laser scatter aggregation, it was found that young smokers had an increased number of small platelet aggregates, which cannot be detected with a conventional aggregometer based on the turbidometric method (Matsuo et al. 1997). This device detects platelet aggregation in the small-aggregate size range by the addition of unfractionated heparin (UFH), and the aggregates are disaggregable in incubation with protamine sulfate. When platelet aggregation induced by UFH at a final concentration of 0.5 U/mL was observed in 36 normal subjects with no history of heparin exposure, 13 had a positive response in excess of 0.5 V of light intensity in the small-aggregate size

range. In chronic hemodialysis patients in whom heparin had been used regularly for many years, a positive response with heparin-induced aggregates was noted in 37 of 59 patients, which was increased compared with that of normal subjects. The light intensity in the small-aggregate size range was enhanced during heparinized dialysis. In patients with a positive heparin response, the intensity of aggregates after heparin was significantly increased compared with that in nonresponders to heparin. Also, we obtained the same results by this system: that enhanced platelet aggregation response to heparin was not inhibited by aspirin or argatroban but was inhibited by anti-glycoprotein IIb/IIIa antibodies or small molecule antagonists. The findings of enhanced platelet aggregation during heparin infusion could be directly obtained without the addition of ADP or TRAP using laser aggregometry (Xiao and Thérout 1998).

LIMITATIONS

This technique can not be applied in whole blood yet but can be used with PRP, washed platelet, or GFP

MODIFICATIONS OF THE METHOD

For functional characterization of an acid platelet aggregation inhibitor and hypotensive phospholipase A₂ from *Bothrops jararacussu* snake venom, Andrião-Escarso et al. (2002) measured platelet aggregation in rabbit blood using a whole blood Lumi-Aggregometer.

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B.2.8**Fibrinogen Receptor Binding****PURPOSE AND RATIONALE**

The assay is used to evaluate the binding characteristics of drugs at the fibrinogen receptor. A constant concentration of the radioligand ^{125}I -fibrinogen (30–50 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of gel-filtered human platelets. If the test drug exhibits any affinity to fibrinogen receptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent the test drug. Platelets are activated with 10 mmol/l ADP to stimulate the ^{125}I -fibrinogen binding at the GPIIb/IIIa receptor.

PROCEDURE**Preparation of Gel-Filtered Platelets**

From a healthy volunteer, 200 ml blood is collected. An aliquot of 8.4 ml blood is mixed with 1.4 ml ACD-buffer in polystyrol tubes and centrifuged at $150 \times g$ for 15 min. The resulting platelet-rich plasma (PRP) is collected, and an aliquot is taken for platelet counting. Then, 10 ml PRP are mixed with 1 ml ACD-buffer (ACD-PRP, pH ~ 6.5); 5 ml portions of ACD-PRP are transferred to plastic tubes and centrifuged at $1500 \times g$ for 15 min. The resulting supernatant is decanted, and each pellet is resuspended in 500 μl Tyrode buffer C. An aliquot is taken for platelet counting to calculate the loss of platelets. The platelet suspension is then transferred to a Sepharose-packed column that has been eluted with approx. 100 ml degassed Tyrode buffer B (2 ml/min). The column is closed and eluted with degassed Tyrode buffer B (2 ml/min). The first platelets appear after 18–20 min and are then collected for 10 min in a closed plastic cup. Gel-filtered platelets (GFP) are set to 4×10^8 platelets/ml with Tyrode buffer B and kept at room temperature until the start of the test (Mousa et al. 1994 and 2001).

EXPERIMENTAL COURSE

For each concentration, samples are tested in triplicate (test tubes No. 72708, Sarstedt). The total volume of each incubation sample is 500 μl . The concentration of ^{125}I -fibrinogen is constant for all samples (10 $\mu\text{g}/500 \mu\text{l}$).

COMPETITION EXPERIMENTS

The competition reaction is characterized by one buffer value (bidistilled water) and various concentrations of non-labeled fibrinogen or test compound.

- 100 μl ^{125}I -fibrinogen
- 100 μl non-labeled fibrinogen or test drug (various concentrations, 10^{-10} – 10^{-3} M)
- 5 μl ADP.

Non-specific-binding: The non-specific binding of ^{125}I -fibrinogen is defined as the radioligand binding in the presence of 10^{-5} M of non-labeled fibrinogen.

The binding reaction is started by adding 250 μl GFP (4×10^8 platelets/ml). The samples are incubated for 30 min at room temperature. Subsequently, a 100 μl aliquot of the incubation sample is transferred to a microtainer tube containing 400 μl glucose solution. The tubes are centrifuged at $1,500 \times g$ for 2 min to separate ^{125}I -fibrinogen bound at the platelet glycoprotein IIb–IIIa receptor from free radioligand. The supernatant is carefully decanted and is allowed to run off for approx. 30 min. Radioactivity of the platelet pellets is counted for 1 min in a gamma counter with an efficiency of 65.3%.

MATERIALS AND SOLUTIONS

See Table 5.

EVALUATION

The quantity of the specific ^{125}I -fibrinogen binding results from the difference between the total and the non-specific binding.

Platelet glycoprotein IIb–IIIa receptor binding is given as fmol ^{125}I -fibrinogen/ 10^8 platelets or ^{125}I -fibrinogen molecules bound per platelet.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{125}I -fibrinogen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K = \frac{K_D^{125}\text{I} \times IC_{50}}{K_D^{125}\text{I} + [^{125}\text{I}]}$$

IC_{50} = concentration of the test drug, which displaces 50% of the specifically glycoprotein IIb–IIIa receptor bound ^{125}I -fibrinogen in the competition experiment.

$[^{125}\text{I}]$ = concentration of ^{125}I -fibrinogen in the competition experiment.

$K_D^{125}\text{I}$ = dissociation constant of ^{125}I -fibrinogen, determined from the saturation experiment.

The K_i -value of the test drug is the concentration, at which 50% of the fibrinogen receptors are occupied by the test drug.

For detailed methodology and evaluations of various mechanisms and agents see the following selected

Table 5 Materials and solutions

| Solutions for platelet preparation | | |
|------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Stock solution I | Citrate | 0.8% |
| | Sodium citrate | 2.2% |
| Stock solution II | NaCl | 120 mM |
| | KCl | 2.8 mM |
| | NaH ₂ PO ₄ | 10.0 mM |
| | HEPES | 10.0 mM |
| ACD-buffer | Stock solution I | 2.45% |
| | + glucose | |
| | + hirudin | 0.06 U/ml |
| Tyrode buffer A | Stock solution II + NaHCO ₃ | 12 mM |
| Tyrode buffer B | Stock solution II | |
| | + NaHCO ₃ | 12 mM |
| | + glucose | 5.5 mM |
| | + bovine albumin | 0.35% |
| Tyrode buffers A and B are degassed by aspiration for approx. 1 h after setting the pH to 7.2. | | |
| Tyrode buffer C | Tyrode buffer B (degassed) | |
| | + apyrase | 40 µg/ml |
| | + hirudin | 0.06 U/ml |
| Chromatography column | Acryl glass column (200 × 170 mm, 30 mm diameter), closed with 3 perlon filters, pore sizes 63, 90 and 230 µm, and gauze 50 µm filled with degassed Sepharose CL2B-suspension (Pharmacia LKB); equilibrated with 500 ml degassed Tyrode buffer A (2 ml/min) | |
| Incubation buffer | | |
| Stock solution | NaCl | 120 mM |
| | KCl | 2.6 mM |
| | NaH ₂ PO ₄ | 0.39 mM |
| | HEPES | 10.0 mM |
| | CaCl ₂ | 0.5 mM |
| Incubation buffer, pH 7.2 | stock solution | |
| | + NaHCO ₃ | 12 mM |
| | + glucose | 5.5 mM |
| | + human albumin | 0.35% |
| Glucose solution (in incubation buffer) | | |
| Radioligand | ¹²⁵ I-fibrinogen specific activity 3.7 Mbq/mg fibrinogen (100 µCi/mg fibrinogen) (Amersham), 1 mg radio-labeled fibrinogen is dissolved in 10 ml incubation buffer | |
| Non-labeled fibrinogen (mw 340000, grade L, Sigma; in bidistilled water) | 10 ⁻³ –10 ⁻¹⁰ M | |
| ADP (in incubation buffer) | 10 µM | |
| Gamma-counter (1282 Compugamma CS, LKB) | | |

references: Bennett, Vilaire (1979); Kornecki et al. (1981); Marguerie et al. (1979 and 1980); Mendelsohn et al. (1990), and Mousa et al. (1994).

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B.2.9

Euglobulin Clot Lysis Time

PURPOSE AND RATIONALE

The euglobulin lysis time is used as an indicator for the influence of compounds on the fibrinolytic activity in rat blood according to Gallimore et al. (1971). The euglobulin fraction of plasma is separated from inhibitors of fibrinolysis by acid precipitation and centrifugation. Euglobulin predominantly consists of plasmin, plasminogen, plasminogen activator and fibrinogen. By addition of thrombin to this fraction, fibrin clots are formed. The lysis time of these clots is determined as a measurement of the activity of activators of fibrinolysis (e. g. plasminogen activators). Thus, compounds that stimulate the release of tissue-type plasminogen activator from the vessel wall can be detected.

PROCEDURE

Rats are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad (37°C). At the same time, the test solution or the vehicle (controls) is administered intravenously or intraperitoneally. Twenty-five minutes later, the animals receive another intraperitoneal injection of 12 mg/kg sodium pentobarbital to keep them in deep narcosis for 45 min.

Plasma Preparation

After the test compound is absorbed, blood is withdrawn from the inferior caval vein exposed by a mid-line excision. Blood (1.8 ml) is removed with a plastic syringe containing 0.2 ml 3.8% sodium citrate solution. The sample is thoroughly mixed, transferred

to a plastic tube and immediately immersed in ice. Plasma is prepared by centrifugation at 2000 g for 10 min at 2°C.

Euglobulin Preparation

A 0.5 ml portion of plasma is added to 9.5 ml of ice-cold distilled water; the pH is brought to 5.3 by the addition of 0.13 ml of 1% acetic acid. The diluted plasma is kept on ice for 10 min, and the precipitated euglobulin fraction is collected by centrifugation at 2000 g for 10 min at 2°C. The supernatant is discharged, and the remaining fluid is removed by drying the tube on a filter paper for 1 min. The euglobulin precipitate is dissolved in 1 ml of 0.12 M sodium acetate solution.

Euglobulin Lysis Assay

Aliquots (0.45 ml) of the euglobulin solution are transferred to test tubes, and 0.05 ml thrombin (Test Thrombin, Behring Werke) (25 U/ml) are added. The tubes are transferred to a water bath at 37°C. The time interval between the addition of thrombin and the complete lysis of the clots is measured. For details see Gallimore et al. (1971) and Singh et al. (2005).

EVALUATION

The lysis time (min) is determined. Euglobulin lysis test (ELT) is shortened when activators of fibrinolysis are increased.

Percent lysis time is calculated in dosage treated groups as compared to controls.

Statistical evaluation is performed by means of the Student's *t*-test.

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B.2.10

Flow Behavior of Erythrocytes

PURPOSE AND RATIONALE

The deformation of erythrocytes is an important rheological phenomenon in blood circulation according to Teitel (1977) and Nash (1990). It allows the passage of normal red cells through capillaries with diameters

smaller than that of the discoid cells and reduces the bulk viscosity of blood flowing in large vessels. In the following test, the initial flow of filtration is taken as a criterion for erythrocyte deformability. A prolonged time of filtration can be due to 2 basic pathologic phenomena: an increased rigidity of the individual red cells or an increased tendency of the cells to aggregate. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified by one (or by the combination) of the following stress factors:

- addition of calcium ions (increase in erythrocyte rigidity)
- addition of lactic acid (decrease in pH value)
- addition of 350–400 mmol NaCl (hyperosmolarity)
- storing the sample for at least 4 h (cellular ageing, depletion of ADP)

The following procedure can be used to evaluate the effect of test compounds on the flow behavior of erythrocytes.

PROCEDURE

Apparatus

Erythrocyte filtrometer MF4 (Fa. Myrenne, 52159 Roetgen, Germany) Membrane filter (Nuclepore Corp.) pore diameter: 5–10 μm, pore density: 4×10^5 pores/cm².

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, or from Wistar rats weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, blood is incubated at 37°C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with K-EDTA (1 mg/ml blood) or heparin (5 IU/ml heparin sodium) and centrifuged at $250 \times g$ for 7 min. The supernatant (plasma) and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in autologous plasma containing 0.25% human albumin, and the haematocrit value is fixed at 10%. The red blood cells are altered by one or several of the stress factors mentioned above.

A sample of 2 ml of the stressed suspension is applied to the filtrometer and the initial flow rate is determined. The filtration curve is plotted automatically.

EVALUATION

The cumulative volume of the filtered suspension is recorded per time unit (10 min)

The slope of the curve is determined at different time intervals.

The initial flow rate (10% of the cell suspension having passed the filter) is recorded.

Statistics

Data of each set are first tested for normal distribution using the Kolmogoroff/Smirnow test. The normal distribution hypothesis is eliminated if the data having a significance level of 5% are not normally distributed. In case that both data sets to be compared are normally distributed, the F-test is applied. The hypothesis of homogeneity of variance of both test series is eliminated when the significance level for homogeneity of variance is 5%. The *t*-test for paired and non-paired data is performed when homogeneity of variance is present. In any case, a paired difference test (for paired data) or the U-test (for non-paired data) is likewise carried out (paired of difference test = Wilcoxon test; U-test = Wilcoxon-Mann-Whitney or Mann-Whitney test, respectively).

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B.2.11

Filterability of Erythrocytes

PURPOSE AND RATIONALE

The Single Erythrocyte Rigidometer (SER) allows the measurement of deformability of individual red blood cells by determining their passage time through a pore under constant shear stress. In this test, the passage times of single erythrocytes through one pore in a synthetic membrane are determined according to Kiesewetter et al. (1982), Roggenkamp et al. (1983), and Seiffge et al. (1986). The pore in the membrane practically represents a capillary with defined diameter and length. The driving pressure is produced by the constant shear stress. The passage of the red blood cells is measured with the help of an electrical device. A constant current of 50–200 nA is applied. When an erythrocyte passes through the pore, the current is interrupted. The test is used to detect compounds that improve filterability of erythrocytes. To simulate decreased red blood cell deformability, the erythrocytes

are artificially modified either by one or by a combination of the following stress factors:

- addition of calcium ions (increase in erythrocyte rigidity)
- addition of lactic acid (decrease in pH value)
- addition of 350–400 mmol NaCl (hyperosmolarity)
- storing the sample for at least 4 h (cellular ageing, depletion of ADP).

PROCEDURE

Apparatus

Single erythrocyte rigidometer (Myrenne, 52159 Roetgen, Germany)

Data: driving pressure: $dp = 70$ Pa (dog, rabbit, rat), $dp = 100$ Pa (man); wall shear stress: $\tau = 3$ Pa.

Single pore membrane: length: $30 \mu\text{m}$; diameter: $3.5 \mu\text{m}$ (rat), $4.0 \mu\text{m}$ (rabbit, dog), $4.5 \mu\text{m}$ (man)

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, the blood samples are incubated at 37°C for 5 or 30 min.

The blood samples are mixed with K-EDTA (1 mg/ml blood) or heparin (5 IE/ml heparin sodium) to prevent clotting. The blood is centrifuged at $250 \times g$ for 7 min. The plasma and the buffy coat are removed and discarded. The packed erythrocytes are re-suspended in filtrated HEPES-buffer containing 0.25% human albumin, and the haematocrit value is fixed to $<1\%$. The red blood cells are altered by one or several stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the measuring device, and the passage time of a population of 250 erythrocytes (t_m) is determined. Cells remaining in the pore for more than 100 ms ($t_m > 100$ ms) lead to a rheological occlusion.

Untreated red blood cell suspensions serve as control.

EVALUATION

The mean passage time of 250 single erythrocytes and the number of rheological occlusions/250 erythrocytes is determined.

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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B.2.12

Erythrocyte Aggregation

PURPOSE AND RATIONALE

The aggregation of red blood cells into rouleaux and from rouleaux into 3-dimensional cell networks is a rheological parameter that decisively influences the flow behavior of blood, especially in disturbed microcirculation. In the following procedure, an apparatus (erythrocyte aggregometer) is used to measure erythrocyte aggregation. The transparent measuring chamber (cone/plate configuration) is transilluminated by light of a defined wave length. The intensity of the transmitted light, which is modified by the aggregation process, is recorded. The method can be used to determine the effect of test compounds on erythrocyte aggregation according to Kiesewetter et al. (1982) and Schmid-Schoenbein et al. (1973).

PROCEDURE

Apparatus

Selective Erythrocyte Rigidometer (Fa. Myrenne, 52159 Roetgen, Germany)

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, or from Wistar rats weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, the blood sample is incubated at 37°C for 5 or 30 min.

Blood is obtained from the test subjects by venipuncture and mixed with K-EDTA (1 mg/ml) or heparin (5 IU/ml heparin sodium) to prevent clotting. Erythrocyte aggregation is determined in whole blood. A sample of 40 µl blood is transferred to the measuring device. The red cells are dispersed at a shear rate of 600/s. After 20 s, flow is switched to stasis, and the extent of erythrocyte aggregation is determined photometrically.

EVALUATION

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes (see above).

REFERENCES AND FURTHER READING

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B.2.13

Determination of Plasma Viscosity

PURPOSE AND RATIONALE

One of the principal methods for measuring viscosity is based on the rate of flow of a liquid through an orifice according to Harkness (1971). In this test, a defined volume of plasma is transferred into a capillary viscometer, and the efflux time required for the plasma to flow from the upper to the lower mark is measured. Using this procedure, the effect of test compounds on the viscosity of blood plasma can be determined. The test can be carried out either *ex vivo* or *in vitro*.

PROCEDURE

Ex Vivo

Beagle dogs weighing 12–20 kg, rabbits weighing

2.0–3.0 kg, or Wistar rats weighing 150–300 g of either sex are used as test animals. Likewise, the test procedure can be performed in humans. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, plasma (obtained as described below) is incubated at 37°C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with 1 mg/ml blood K⁺-EDTA or heparin sodium (5 IU/ml blood) and centrifuged at 250 × g for 5 min. The supernatant (plasma) is removed, and a sample of 0.9 ml plasma is transferred into a capillary viscometer (Coulter Harkness, Coulter Electr., LTD, England) provided with a glass capillary of 0.5 mm inside diameter. The temperature during measurement is 37°C. The flow time, *t*, required for the plasma to flow through the capillary is measured. Untreated plasma serves as control.

EVALUATION

The viscosity of each sample can be determined using the following formula:

$$\eta = K \times t \times \rho$$

where η = viscosity of plasma, K = calibration constant of viscometer, t = flow time of 0.9 ml plasma, and ρ = density of plasma.

The change in viscosity relative to the control group is determined.

Statistical evaluation is carried out using the Student's *t*-test.

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B.3

In Vitro Models of Thrombosis

PURPOSE AND RATIONALE

There is abundant evidence suggesting that platelets play a pivotal role in the pathogenesis of arterial

thrombotic disorders, including unstable angina (UA), myocardial infarction (MI) and stroke. The underlying pathophysiological mechanism of these processes has been recognized as the disruption or erosion of a vulnerable atherosclerotic plaque leading to local platelet adhesion and subsequent formation of partially or completely occlusive platelet thrombi.

The specific platelet surface receptors that support these initial adhesive interactions are determined by the local fluid dynamic conditions of the vasculature and the extracellular matrix constituents exposed at the sites of vascular injury. Konstantopoulos et al. (1998) and Alveriadou et al. (1993) demonstrated that under high shear conditions, the adhesion of platelets to exposed subendothelial surfaces of atherosclerotic or injured vessels presenting collagen and von Willebrand factor (vWF) is primarily mediated by the platelet glycoprotein (GP)Ib/IX/V complex. This primary adhesion to the matrix activates platelets, leading ultimately to platelet aggregation mediated predominantly by the binding of adhesive proteins such as fibrinogen and vWF to GPIIb/IIIa. In addition, direct platelet aggregation in the bulk phase under conditions of abnormally elevated fluid shear stresses, analogous to those occurring in atherosclerotic or constricted arterial vessels, as shown by Turitto (1982), may be important. Shear-induced platelet aggregation is dependent upon the availability of vWF and the presence of both GPIb/IX and GPIIb/IIIa on the platelet membrane. It has been postulated that at high shear stress conditions, the interaction of vWF with the GPIb/IX complex is the initial event leading to platelet activation, which also triggers the binding of vWF to GPIIb/IIIa to induce platelet aggregate formation.

A variety of methods have been utilized to assess the *ex vivo* and/or *in vitro* efficacy of platelet antagonists, including photometric aggregometry, whole blood electrical aggregometry and particle counter methods, as described in the above segments. In photometric aggregometry, a sample is placed in a stirred cuvette in the optical light path between a light source and a light detector. Aggregate formation is monitored by a decrease in turbidity, and the extent of aggregation is measured as percent of maximal light transmission. The major disadvantage of this technique is that it cannot be applied in whole blood since the presence of erythrocytes interferes with the optical responses. Furthermore, it is insensitive to the formation of small aggregates. Particle counters are used to quantitate the size and the number of particles suspended in an electrolyte solution by monitoring the electrical current between two electrodes immersed in the solution. Ag-

gregation in this system is quantitated by counting the platelets before and after stimulation, and it is usually expressed as a percentage of the initial count, as shown by Jen and McIntire (1984). However, the disadvantage of this technique is that it cannot distinguish platelets and platelet aggregates from other blood cells of the same size. Thus, one is limited to counting only a fraction of single platelets, as well as aggregates that are much larger than erythrocytes and leukocytes. The technique of electrical aggregometry allows the detection of platelet aggregates as they attach to electrodes immersed in a stirred cuvette of whole blood or platelet suspensions. Such an attachment results in a decrease in conductance between the two electrodes that can be quantitated in units of electrical resistance. However, a disadvantage of this method is that it is not sensitive in the detection of small aggregates, as demonstrated by Sweeney et al. (1989).

This segment discusses two complementary *in vitro* flow models of thrombosis that can be used to accurately quantify platelet aggregation in anticoagulated whole blood specimens and to evaluate the inhibitory efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure direct shear-induced platelet aggregation in the bulk phase, as demonstrated by Konstantopolous et al. (1995); and (2) a parallel-plate perfusion chamber coupled with a computerized videomicroscopy system to quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood flowing over an immobilized substrate (e.g. collagen I), as shown by Konstantopolous et al. (1995), and Mousa et al. (2002). Furthermore, Mousa et al. (2002) demonstrated a third *in vitro* flow assay in which surface-anchored platelets are pre-incubated with a GPIIb/IIIa antagonist, and unbound drug is washed away prior to the perfusion of THP-1 monocytic cells, thereby enabling one to distinguish agents with markedly distinct affinities and receptor-bound lifetimes.

PROCEDURE

Isolation of Human Platelets

The steps described in subsequent subheadings outline the procedure for isolation and purification of platelets from whole blood obtained by venipuncture from human volunteers. Obtain blood sample by venipuncture from an antecubital vein into polypropylene syringes containing either sodium citrate (0.38% final concentration) or heparin (10 U/ml final concentration). Centrifuge anticoagulated whole blood at $160 \times g$ for 15 min to prepare platelet-rich plasma (PRP).

Isolation of Washed Platelets

PRP specimens are subjected to a further centrifugation (1,100 *g* for 15 min) in the presence of 2 μM PGE₁ (Evangelista et al. 1996).

The platelet pellet is resuspended in HEPES-Tyrode buffer containing 5 mM EGTA and 2 μM PGE₁.

Platelets are then washed via centrifugation (1,100 *g* for 10 min), resuspended at 2×10^8 /mL in HEPES-Tyrode buffer, and kept at room temperature for no longer than 4 hours before use in aggregation/adhesion assays.

MATERIALS

- Anticoagulant solution (sodium citrate, porcine heparin, PPACK etc.)
- Fluorescently labeled platelet-specific antibody
- Dulbecco's phosphate-buffered saline (D-PBS) (with and without Ca²⁺/Mg²⁺).
- Formaldehyde
- Type I collagen, from bovine Achille's tendon
- 0.5 mol/L glacial acetic acid in water
- Glass coverslips (24 × 50 mm; Corning; Corning, NY)
- Silicone sheeting (gasket) (0.005-in or 0.010-in thickness; Specialty Manufacturing Inc; Saginaw, MI)
- Quinacrine dihydrochloride
- Prostaglandin E₁ (PGE₁) and EGTA
- Thrombin
- Bovine serum albumin
- HEPES-Tyrode buffer (129 mM NaCl, 9.9 mM NaHCO₃, 2.8 mM KCl, 0.8 mM K₂PO₄, 0.8 mM MgCl₂ · 6H₂O, 1 mM CaCl₂, 10 mM HEPES, 5.6 mM dextrose)
- 3-aminopropyltriethoxysilane (APES)
- Acetone
- 70% nitric acid in water
- THP-1 monocytic cells
- Platelet antagonists such as abciximab

EVALUATIONS

The methods described below (Sections 2.1–2.3) outline different dynamic adhesion/aggregation assays used to assess the *in vitro* and/or *ex vivo* efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure shear-induced platelet-platelet aggregation in the bulk phase, (2) a perfusion chamber coupled with a computerized videomicroscopy system to visualize in real time and quantify (a) the adhesion and subsequent aggregation of platelets flowing over an

immobilized substrate (e.g. extracellular matrix protein) and (b) free-flowing monocytic cell adhesion to immobilized platelets.

REFERENCES AND FURTHER READING

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B.3.1**Cone-and-Plate Viscometry Under Shear-Flow Cytometry****PURPOSE AND RATIONALE**

The cone-and-plate viscometer is an *in vitro* flow model used to investigate the effects of bulk fluid shear stress on suspended cells. Anticoagulated whole blood specimens (or isolated cell suspensions) are placed between the two platens (both of stainless steel) of the viscometer. Rotation of the upper conical platen causes a well-defined and uniform shearing stress to be applied to the entire fluid medium, as described by Konstantopolous et al. (1998). The shear rate (γ) in this system can be readily calculated from the cone angle and the speed of the cone using the formula:

$$\gamma = \left(\frac{2\pi\omega}{60\theta_{cp}} \right)$$

where γ is the shear rate in sec^{-1} , ω is the cone rotational rate in revolutions per minute (rev/min) and θ_{cp} is the cone angle in radians. The latter is typically in the range of 0.3 to 1.0° . The shear stress, τ , is proportional to shear rate, γ , as shown by: $\tau = \mu \cdot \gamma$, where μ is the viscosity of the cell suspension (the viscosity of anticoagulated whole blood is ~ 0.04 cp at 37°C). This type of rotational viscometer is capable of generating shear stresses from ~ 2 dyn/cm² (venous level) to greater than 200 dyn/cm² (stenotic arteries).

PROCEDURE

Single platelets and platelet aggregates generated upon shear exposure of blood specimens are differentiated from other blood cells on the basis of their characteristic forward-scatter and fluorescence (by the use of fluorophore-conjugated platelet specific antibodies) profiles by flow cytometry, as described by Konstantopoulos et al. (1995). This technique requires no washing or centrifugation steps that may induce artifactual platelet activation, and it allows the study of platelet function in the presence of other blood elements. The procedure used to quantify platelet aggregation induced by shear stress is as follows:

- Incubate anticoagulated whole blood with platelet antagonist or vehicle (control) at 37°C for 10 min.
- Place a blood specimen (typically ~ 500 μl) on the stationary platen of a cone-and-plate viscometer maintained at 37°C .
- Take a small aliquot (~ 3 μl) from the pre-sheared blood sample, fix it with 1% formaldehyde in D-PBS (~ 30 μl).
- Expose the blood specimen, in the presence or absence of a platelet antagonist, to well-defined shear levels (typically 4000 sec^{-1} to induce significant platelet aggregation in the absence of a platelet antagonist) for prescribed periods of time (typically 30 to 60 sec).
- Take a small aliquot (~ 3 μl) from the sheared blood specimen, and immediately fix it with 1% formaldehyde in D-PBS (~ 30 μl).
- Incubate the fixed blood samples with a saturating concentration of a fluorescently labeled platelet-specific antibody, such as anti-GPIIb(6D1)-FITC, for 30 min in the dark.
- Dilute specimens with 2 ml of 1% formaldehyde, and analyze them by flow cytometry.

Flow cytometric analysis is used to distinguish platelets from other blood cells on the basis of their characteristic forward scatter and fluorescence profiles, as shown in Fig. 1. Data acquisition is then

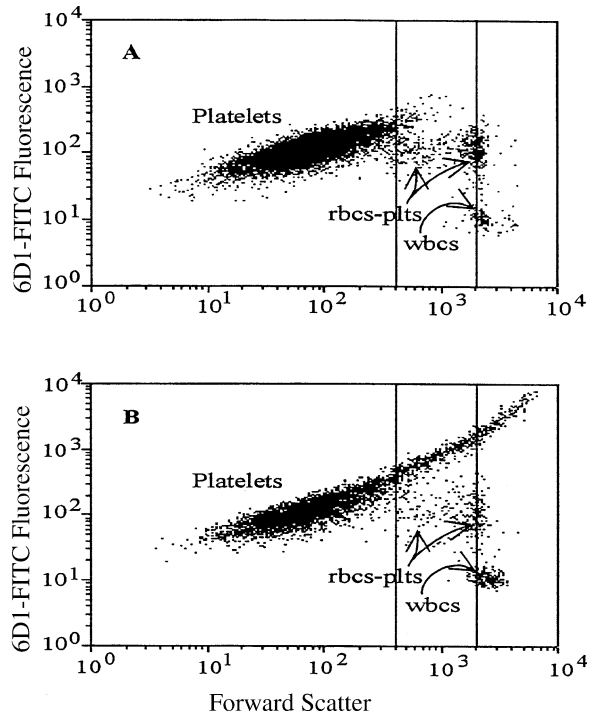


Figure 1 Quantification of shear-induced platelet aggregation by flow cytometry. Panel A corresponds to an unshattered blood specimen. Panel B corresponds to a blood specimen that has been subjected to a pathologically high level of shear stress for 30 sec. As can be seen in the figure, there are three distinct cell populations. The upper population consists of platelets and platelet aggregates. The “rbc-plts” population corresponds to platelets associated with erythrocytes and leukocytes. The “wbcs” population consists of some leukocytes that have elevated levels of FITC autofluorescence. The left vertical line separates single platelets (≤ 4.5 μm in diameter) from platelet aggregates, whereas the right vertical line separates “small” from “large” platelet aggregates. The latter were defined to be larger than 10 μm in equivalent sphere diameter

carried out on each sample for a set period (usually 100 sec), thereby allowing equal volumes for both the pre-sheared and sheared specimens to be achieved. As a result, the percent platelet aggregation can be determined by the disappearance of single platelets into the platelet aggregate region using the formula: $\% \text{ Platelet Aggregation} = (1 - N_s/N_c \times 100)$, where N_s represents the single platelet population of the sheared specimen and N_c represents the single platelet population of the pre-sheared specimen. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be readily determined.

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Konstantopoulos K, Kukreti S, McIntire LV (1998) Biomechanics of cell interactions in shear fields. *Adv Drug Delivery Rev* 33:141–164

B.3.2

Platelet Adhesion and Aggregation Under Dynamic Shear

PURPOSE AND RATIONALE

The steps described and outlined an *in vitro* flow model of platelet thrombus formation, which can be used to evaluate the *ex vivo* and/or *in vitro* efficacy of platelet antagonists. Thrombus formation may be initiated by platelet adhesion from rapidly flowing blood onto exposed subendothelial surfaces of injured vessels containing collagen and von Willebrand factor (vWF), with subsequent platelet activation and aggregation. Konstantopolous et al. (1995) described the use of a parallel-plate flow chamber that provides a controlled and well-defined flow environment based on the chamber geometry and the flow rate through the chamber. The wall shear stress, τ_w , assuming a Newtonian and incompressible fluid, can be calculated using the formula:

$$\tau_w = \frac{6\mu Q}{wh^2}$$

where Q is the volumetric flow rate, μ is the viscosity of the flowing fluid, h is the channel height, and b is the channel width. A flow chamber typically consists of a transparent polycarbonate block, a gasket whose thickness determines the channel depth, and a glass coverslip coated with an extracellular matrix protein such as type I fibrillar collagen. The apparatus is held together by vacuum. Shear stress is generated by flowing fluid (e.g. anticoagulated whole blood or isolated cell suspensions) through the chamber over the immobilized substrate under controlled kinematic conditions using a syringe pump. Mousa et al. (2002) combined the parallel-plate flow chamber with a computerized epi-fluorescence videomicroscopy system that enables one to visualize in real time and separately quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood (or isolated platelet suspensions) flowing over an immobilized substrate.

PROCEDURE

Preparation of Collagen-Coated Surfaces

- Dissolve 500 mg collagen type I from bovine

Achille's tendon into 200 ml of 0.5 mol/L acetic acid in water, pH 2.8.

- Homogenize for 3 hours.
- Centrifuge the homogenate at 200g for 10 min, collect supernatant, and measure collagen concentration by a modified Lowry analysis.
- Coat glass coverslips with 200 μ l of fibrillar collagen I suspension on all but first 10 mm of the slide length (coated area = 12.7 \times 23), and place in a humid environment at 37°C for 45 min.
- Rinse excess collagen with 10 ml of D-PBS maintained at 37°C before assembly into the flow chamber (Folie et al. 1988).

Platelet Perfusion Studies

- Add the fluorescent dye quinacrine dihydrochloride to anticoagulated whole blood samples at a final concentration of 10 μ M immediately after blood collection.
- Prior to the perfusion experiment, incubate blood with either a platelet antagonist or vehicle (control) at 37°C for 10 min.
- Perfuse anticoagulated whole blood through the flow chamber for 1 min at wall shear rates ranging from 100 sec^{-1} (typical of venous circulation) to 1500 sec^{-1} (mimicking partially constricted arteries) for prescribed periods of time (e.g. 1 min). Platelet-substrate interactions are monitored in real time using an inverted microscope equipped with an epifluorescent illumination attachment and silicon-intensified target video camera, and recorded on videotape. The microscope stage and flow chamber are maintained at 37°C by an incubator heating module and incubator enclosure during the experiment.

EVALUATION

Videotaped images are digitized and computer analyzed at 5, 15 and 60 sec for each perfusion experiment. The number of adherent individual platelets in the microscopic field of view during the initial 15 sec of flow is determined by image processing and used as the measurement of platelet adhesion that initiates platelet thrombus formation. The number of platelets in each individual thrombus is calculated as the total thrombus intensity (area \times fluorescence intensity) divided by the average intensity of single platelets determined in the 5-sec images. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be de-

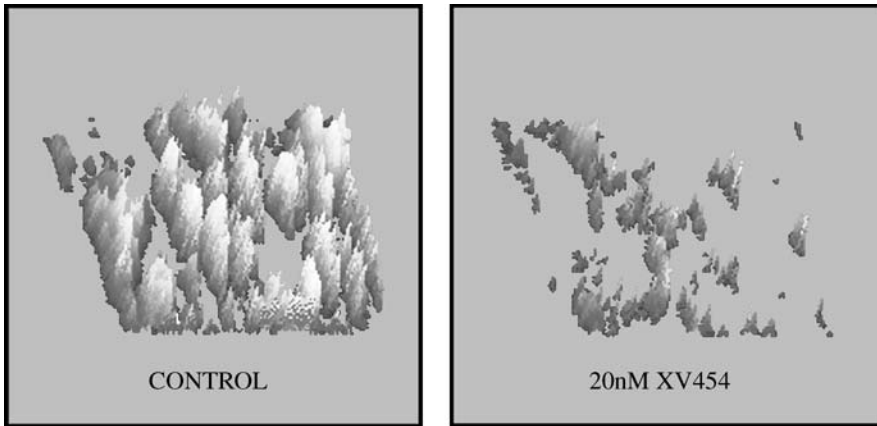


Figure 2 Three-dimensional computer-generated representation of platelet adhesion and subsequent aggregation on collagen I/von Willebrand factor from normal heparinized blood perfused in the absence (control) or presence of a GPIIb/IIIa antagonist (XV454) at 37°C for 1 min at 1,500 sec⁻¹

terminated (Fig. 2). Along these lines, any potential inhibitory effects of a platelet antagonist on platelet adhesion can be readily assessed.

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B.3.3

Cell Adhesion to Immobilized Platelets: Parallel-Plate Flow Chamber

PURPOSE AND RATIONALE

In this assay, immobilized platelets are pretreated with a GPIIb/IIIa antagonist, and any unbound drug is washed away before the perfusion of monocytic THP-1 cells. Mousa et al. (1998) demonstrated that agents with slow platelet off-rates such as XV454 ($t_{1/2}$ of dissociation = 110 min; K_d = 1 nM) and abciximab ($t_{1/2}$ of dissociation = 40 min; K_d = 9.0 nM) that are distributed predominantly as receptor-bound entities with little unbound in the plasma, can effectively block these heterotypic interactions as shown by Abulencia et al. (2001) and by Mousa et al. (2002). In contrast, agents with relatively fast platelet dissociation rates such as orbofiban ($t_{1/2}$ of dissociation = 0.2 min; K_d > 110 nM), whose antiplatelet efficacy depends on the plasma concentration of the active drug, do not exhibit any inhibitory effects, as described by Mousa et al. (2002).

PROCEDURE

Preparation of 3-Aminopropyltriethoxysilane (APES)-Treated Glass Slides

- Soak glass coverslips overnight in 70% nitric acid.
- Wash coverslips with tap water for 4 hours.
- Dry coverslips by washing once with acetone, followed by immersion in a 4% solution of APES in acetone for 2 min.
- Repeat the step above, followed by a final rinse of the glass coverslips with acetone.
- Wash coverslips three times with water, and allow them to dry overnight.

Immobilization of Platelets on 3-APES-Treated Glass Slides

- Layer washed platelets or platelet-rich plasma (2×10^8 cells/ml) on the surface of a coverslip at $\sim 30 \mu\text{l}/\text{cm}^2$.
- Allow platelets to bind to APES-treated coverslip in a humid environment at 37°C for 30 min.

Monocytic THP-1 Cell-Platelet Adhesion Assay

- Assemble the platelet-coated coverslip on a parallel-plate flow chamber, which is then mounted on the stage of an inverted microscope equipped with a CCD camera connected to a VCR and TV monitor.
- Perfuse the antiplatelet antagonist at the desirable concentration or vehicle (control) over surface-bound platelets, and incubate for 10 min. The extent of platelet activation can be further modulated by the presence of chemical agonists such as thrombin (0.02–2 U/ml) during the 10 min incubation. The microscope stage and flow chamber are maintained

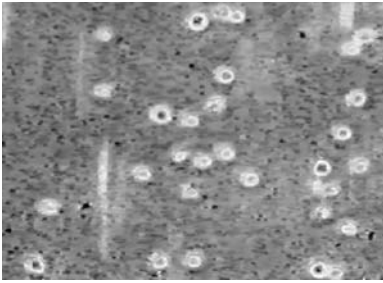


Figure 3 Phase-contrast photomicrograph of THP-1 cells (phase bright objects) attached to a layer of thrombin-treated platelets (phase dark objects) after THP-1 cell perfusion for 3 min at a shear stress level of 1.5 dyn/cm^2

at 37°C by an incubator heating module and incubator enclosure during the experiment.

- In some experiments, unbound platelet antagonist is removed by a brief washing step (4 min) prior to the perfusion of the cells of interest over the platelet layer. In others, the desirable concentration of the platelet antagonist is continuously maintained in the perfusion buffer during the entire course of the experiment.
- Perfuse cells (e. g. THP-1 monocytic cells, leukocytes, tumor cells, protein-coated beads, etc.) over surface-bound platelets, either in the presence or absence of a platelet antagonist (see above), at the desirable flow rate for prescribed periods of time. THP-1 cell binding to immobilized platelets is monitored in real time and recorded on videotape.
- Determine the extent of THP-1 cell tethering, rolling and stationary adhesion to immobilized platelets, as well as the average velocity of rolling THP-1 cells. By comparing the corresponding extents of THP-1 cell tethering, rolling and stationary adhesion to immobilized platelets in the presence and absence of a platelet antagonist (Fig. 3), its antiplatelet efficacy can be determined as shown by Mousa et al. (1995).

EVALUATION

Low-speed centrifugation results in the separation of platelets (top layer) from larger and more dense cells such as leukocytes and erythrocytes (bottom layer). To minimize leukocyte contamination in PRP specimens, slowly aspirate the uppermost two thirds of the platelet layer. Furthermore, certain rare platelet disorders, such as Bernard-Soulier Syndrome (BSS), are characterized by larger than normal platelets which must therefore be isolated by allowing whole blood to gravity separate for 2 hours post-venipuncture.

The mechanical force most relevant to platelet-mediated thrombosis is shear stress. The normal time-averaged levels of venous and arterial shear stresses range between $1\text{--}5 \text{ dyn/cm}^2$ and $6\text{--}40 \text{ dyn/cm}^2$, respectively. However, fluid shear stress may reach levels well over 200 dyn/cm^2 in small arteries and arterioles partially obstructed by atherosclerosis or vascular spasm. The cone-and-plate viscometer and parallel-plate flow chamber are two of the most common devices used to simulate fluid mechanical shearing stress conditions in blood vessels.

Due to the large concentration of platelets and erythrocytes in whole blood, small aliquots ($\sim 3 \mu\text{l}$) of pre-sheared and post-sheared specimens must be obtained and processed prior to the flow cytometric analysis. This will minimize an artifact produced as a platelet and an erythrocyte pass through the light beam of a flow cytometer at the same time.

The “rbc-plts” population represents 3–5% of the displayed cells. A small fraction ($\sim 5\%$) of this population seems to be leukocyte-platelet aggregates, as evidenced by the use of an anti-CD45 monoclonal antibody. The remaining events correspond to erythrocytes associated with platelets. However, it appears that the majority of the latter population is an artifact generated by the simultaneous passage of a platelet and an erythrocyte through the beam of a flow cytometer. This concept is corroborated by the fact that further dilution of pre-sheared and sheared blood specimens and/or reduction of the sample flow rate during the flow cytometric analysis results in a dramatic relative decrease of the “rbc-plts” population.

The collagen density remaining on glass coverslips after D-PBS rinsing can be measured by the difference in weight of 20 clean uncoated slides versus 20 collagen-treated slides.

Experiments are optimally monitored $\sim 100\text{--}200 \mu\text{m}$ downstream from the collagen/glass interface using a $60\times$ FLUOR objective and $1\times$ projection lens, which gives a $3.2\times 10^4 \mu\text{m}^2$ field of view. A field of view closer to the interface may lead to non-reproducible results due to variations in the collagen layering in that region. In contrast, positions farther downstream are avoided in order to minimize the effects of upstream platelet adhesion and subsequent aggregation on both the fluid dynamic environment as well as bulk platelet concentration.

The digitization of a background image (at the onset of perfusion prior to platelet adhesion to the collagen I surface) and its subtraction from a subsequent image acquired 5 sec after an initial platelet adhesion event allows the determination of the fluorescence in-

tensity emitted by a single platelet. The intensity level of each single platelet is measured as a mean gray level between 0 (black) and 255 (white) through the use of an image processing software (e. g. OPTIMAS; Agris-Schoen Vision Systems, Alexandria, VA), and is multiplied by its corresponding area (total number of pixels covered by each single platelet). The aforementioned products are then averaged for all single platelet events detected at the 5-sec time point, thus enabling us to calculate the average intensity of single platelets.

A single field of view ($10 \times 0.55 \text{ mm}^2$) is monitored during the 3 min period of the experiment; at the end, five additional fields of view (0.55 mm^2) are monitored for 15 sec each. The following parameters can be quantified: (a) the number of total interacting cells per mm^2 during the entire 3-min perfusion experiment; (b) the number of stationary interacting cells per mm^2 after 3 min of shear flow; (c) the percentage of total interacting cells that are stationary after 3 min of shear flow; and (d) the average rolling velocity ($\mu\text{m}/\text{sec}$) of interacting cells. The number of interacting cells per mm^2 is determined manually by reviewing the videotapes. Stationary interacting cells per mm^2 are considered as those that move <1-cell radius within 10 sec at the end of the 3-min attachment assay. To quantify their number, images can be digitized from a videotape recorder using an imaging software package (e. g. OPTIMAS). Rolling velocities can be computed as the distance traveled by the centroid of the rolling THP-1 cell divided by the time interval using image processing.

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B.4

In Vivo or Ex Vivo Models

PURPOSE AND RATIONALE

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. He proposed three factors responsible for thrombogenesis: obstruction of blood flow, changes in the

properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two or all three factors of Virchow's triad. Therefore, they differ with respect to the prothrombotic challenge—either stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, laser light), insertion of foreign surface, or injection of a prothrombotic factor, and they differ with respect to the vessel type and with respect to the animal species.

Roughly, two types of models can be differentiated (Didisheim 1972; Kaiser et al. 1999; Mousa et al. 1998; Perzborn et al. 2005): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor, resulting in fibrin-rich “red” venous type thrombi; or (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis, resulting in platelet-rich “white” mural thrombi. But the differentiation is not strict because platelets and the coagulation system influence each other. Drugs preventing fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in humans, namely atherosclerosis or thrombophilias, are not included in the models. Thus, any model is limited regarding its clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. In spite of these limitations, animal models predict clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a recent review by Leadley et al. (2000). Furthermore, the clinical usefulness of an antithrombotic drug is determined by its safety/efficacy ratio regarding the bleeding risk. Assessment of a parameter of the hemostatic system should therefore be included in the models if possible.

The development of antithrombotic agents requires pre-clinical assessment of the biochemical and pharmacologic effects of these drugs. It is important to note that the second- and third-generation antithrombotic drugs are devoid of *in vitro* anticoagulant effects, yet *in vivo*, by virtue of endogenous interactions, these drugs produce potent antithrombotic actions. The initial belief that an antithrombotic drug must exhibit *in vitro* anticoagulant activity is no longer valid. This important scientific observation has been possible only because of the availability of animal models.

Several animal models utilizing species such as rats, rabbits, dogs, pigs and monkeys have been made available for routine use. Other animal species such as the hamster, mouse, cat and guinea pig have also been uti-

lized. Species variations are an important consideration in selecting a model and interpreting the results as these variations can result in different antithrombotic effects. Rats and rabbits are the most commonly used species in which both arterial and venous thrombosis has been investigated. Both pharmacologic and mechanical means have been used to produce a thrombogenic effect in these models. Both rat and rabbit models for studying bleeding effects of drugs have also been developed. The rabbit ear blood loss model is most commonly used to test the hemorrhagic effect of drugs. The rat tail bleeding models have also been utilized for the study of several antithrombotic drugs.

These animal models have been well established and can be used for the development of antithrombotic drugs. It is also possible to use the standardized bleeding and thrombosis models to predict the safety and efficacy of drugs. Thus, in addition to the evaluation of *in vitro* potency, the endogenous effect of antithrombotic drugs can also be investigated. Such standardized methods can be recommended for inclusion in pharmacopoeial screening procedures. Numerous models have now been developed to mimic a variety of clinical conditions where antiplatelet and antithrombotic drugs are used, including myocardial infarction, stroke, cardiopulmonary bypass, trauma, peripheral vascular diseases and restenosis. While dog and primate models are relatively expensive, they have also provided useful information on the pharmacokinetics and pharmacodynamics of antithrombotic drugs. The primate models, in particular, have been extremely useful, as the hemostatic pathways in these species are comparable to those in humans. The development of such agents as the specific glycoprotein IIb/IIIa inhibitor antibodies relies largely on these models. These models are, however, of pivotal value in the development of antithrombotic drugs and provide extremely useful data on the safety and efficacy of new drugs developed for human usage.

PROCEDURE

Animal Models of Thrombosis

In most animal models of thrombosis, healthy animals are challenged with thrombogenic (pathophysiologic) stimuli and/or physical stimuli to produce thrombotic or occlusive conditions. These models are useful for the screening of antithrombotic drugs.

I. Stasis-Thrombosis Model: Since its introduction by Wessler et al. (1959), the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic

agents. This model has also been adapted for use in rats (Meuleman et al. 1991). In the stasis thrombosis model, a hypercoagulable state is mimicked by administration of one of a number of thrombogenic challenges, including human serum (Carrie et al. 1994), thromboplastin (Walenga et al. 1986), activated prothrombin complex concentrates (Vlasuk et al. 1991), factor Xa (Millet et al. 1994) and recombinant relipidated tissue factor (Callas et al. 1995). This administration serves to produce a hypercoagulable state. Diminution of blood flow achieved by ligating the ends of the vessel segments serves to augment the prothrombotic environment. The thrombogenic environment produced in this model simulates venous thrombosis where both blood flow and the activation of coagulation play a role in the development of a thrombus.

II. Models Based on Vessel Wall Damage: The formation of a thrombus is not solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a non-thrombogenic surface over which the blood flows. Disruption of the endothelium not only limits the beneficial effects enumerated above, but also exposes subendothelial tissue factor and collagen that serve to activate the coagulation and platelet aggregation processes, respectively. Endothelial damage can be induced experimentally by physical means (clamping, catheter), chemical means (FITC, Rose Bengal, ferrous chloride), thermal injury or electrolytic injury.

EVALUATION

Each setting in the design of an animal model can answer specific question in relation to certain thrombotic disorders in human. However, the ultimate model of human thrombosis is in human.

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B.4.1

Stenosis- and Mechanical Injury-Induced Coronary Thrombosis: Folts Model

PURPOSE AND RATIONALE

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina, acute myocardial infarction or sudden death. Treatment with angioplasty, thrombolysis, or by-pass grafts can expose new thrombogenic surfaces and re-thrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts and Rowe (1974) developed the model of periodic acute platelet thrombosis and cyclic flow reductions (CFRs) in stenosed canine coronary arteries. Uchida et al. described a similar model in 1975. The model includes various aspects of unstable angina pectoris (i.e. critical stenosis, vascular damage, downstream vasospasm induced by vasoconstrictors released or generated by platelets).

The cyclic variations in coronary blood flow are a result of acute platelet thrombi that may occlude the vessel but that either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. They are not a result of vasospasm (Folts et al. 1982). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases but its effect is limited. Similarly, CFRs in the Folts model are abolished by aspirin but the effect can be reversed by increases in catecholamines and shear forces (Folts and Rowe 1988). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (Folts 1991).

Five different protocols are described in the following section for the induction of coronary thrombosis.

Coronary Thrombosis Induced by Stenosis

The described preparations are characterized by episodic, spontaneous decreases in coronary blood flow interrupted by restorations of blood flow. CFRs, which are alterations in coronary blood flow, are associated with transient platelet aggregation at the site of the coronary constriction and abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage of the vessel wall is produced by placing a hemostatic clamp on the coronary artery; a fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder upon the damaged part of the vessel. In dogs, the stenosis is critical, i.e. the reactive hyperemic response to a 10-sec occlusion is abolished (protocol 1); in pigs, the stenosis is subcritical, i.e. there is a partial reactive hyperemia left (Just and Schönafinger 1991; protocol 2).

For some animals, especially for young dogs, damage of the vessel wall and stenosis is not sufficient to induce thrombotic cyclic flow variations. In these cases, an additional activation of platelets by infusion of epinephrine (protocol 3) is required, leading to the formation of measurable thrombi. In another preparation (protocol 4), thrombus formation is induced by subcritical stenosis without prior clamping of the artery and infusion of platelet activating factor (PAF), according to the model described by Apprill et al. (1985). In addition to these protocols, coronary spasms induced by released platelet components can influence coronary blood flow. Therefore, this model includes the main pathological factors of unstable angina pectoris.

Coronary Thrombosis Induced by Electrical Stimulation

In this preparation, coronary thrombosis is induced by delivery of low-amperage electrical current to the inti-

mal surface of the artery, according to the method described by Romson et al. (1980a). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours (protocol 5). As a consequence of this time course, the thrombi formed are of the mixed type and contain more fibrin than the platelet thrombi with critical stenosis.

PROCEDURE

Coronary Thrombosis Induced by Stenosis

Protocol 1: Critical Stenosis Dogs of either sex weighing 15–40 kg, at least 8 months of age, are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and continuous infusion of approx. 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space, the pericardium is opened and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LCX to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 2-mm hemostatic clamp for 5 sec. A small cylindrical plastic constrictor, 2–4 mm in length and with an internal diameter of 1.2–1.8 mm (depending on the size of the LCX) is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (2–5 times) until the appropriate narrowing of the vessel is achieved, and cyclic flow variations are observed. In case of an occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site.

Only dogs with regularly repeated CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2–4×60 min and compared to pre-treatment values.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

Protocol 2: Subcritical Stenosis Male castrated pigs (German landrace, weighing 20–40 kg) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.) and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a posi-

tive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space, the pericardium is opened and the left descending coronary artery (LAD) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LAD to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 1-mm hemostatic clamp for 5 sec. A small cylindrical plastic constrictor, 2 mm in length, is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved, which produces cyclic flow reductions. CFRs are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization does not occur spontaneously, the formed thrombus is released at reduction of blood flow by shortly lifting the vessel with forceps.

Only pigs with regularly repeated CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2×60 min and compared to pre-treatment values.

Protocol 3: Stenosis+Epinephrine Infusion If protocol 1 does not lead to CFRs, additionally epinephrine (0.2 µg/kg/min) is infused into a peripheral vein for twice over 60 minutes (60 min before and 60 min following drug administration). CFRs are registered and compared in the 60 min post-drug phase to the 60 min pre-drug phase.

Protocol 4: Stenosis+PAF Infusion The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus formation (subcritical stenosis). For the induction of CFRs, PAF (C 16-PAF, Bachem) (0.2 nmol/kg/min) is infused into a cannulated lateral branch of the coronary artery.

After 30 min, PAF infusion is terminated and blood flow returns to its normal, continuous course. Thirty minutes later, the test substance is concomitantly administered and a second PAF infusion is started for 30 min.

CFRs are registered and compared in the drug-treated, second PAF phase to the pre-drug, first PAF phase.

Coronary Thrombosis Induced by Electrical Stimulation

Protocol 5 The LCX is punctuated distal to the flow probe with a chrome-vanadium-steel electrode (3-

mm length, 1-mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a Teflon coated wire to a 9 V battery, a potentiometer and an amperemeter. A disc electrode (cathode) is secured to a subcutaneous thoracic muscle layer to complete the electrical circuit. The intima is stimulated with 150 μ A for 6 hours. During this time, an occluding thrombosis is gradually formed.

The test substance or the vehicle (control) is administered either at the start of the electrical stimulation or 30 min following the start.

The time interval until the thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) are determined.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

For all protocols, the following preparations and measurements are performed:

- To measure peripheral arterial blood pressure (BP

[mm Hg]), the right femoral artery is cannulated and connected to a Statham pressure transducer.

- Left ventricular pressure (LVP [mm Hg]) is determined by inserting a microtip-catheter via the carotid artery retrogradely.
- Left ventricular end-diastolic pressure (LVEDP [mm Hg]) is evaluated through sensitive amplification of the LVP.
- Contractility (LV dp/dt max [mm Hg/s]) is determined from the initial slope of the LVP curve.
- Heart rate (min^{-1}) is determined from the pulsatile blood pressure curve.
- The ECG is recorded in lead II.
- Arterial pH and concentrations of blood gases are kept at physiological levels by adjusting respiration and infusion of sodium bicarbonate.
- Blood hematocrit values (37%–40%) and number of erythrocytes are kept constant by infusion of oxy-polygelatine in dogs and electrolyte solution in pigs.
- Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic regulation of temperature.
- Template buccal mucosal bleeding time is carried out using the Simplate^â device.

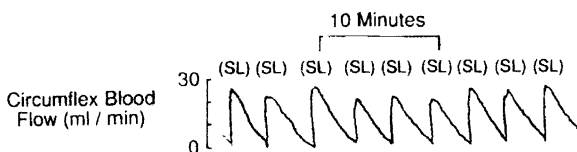
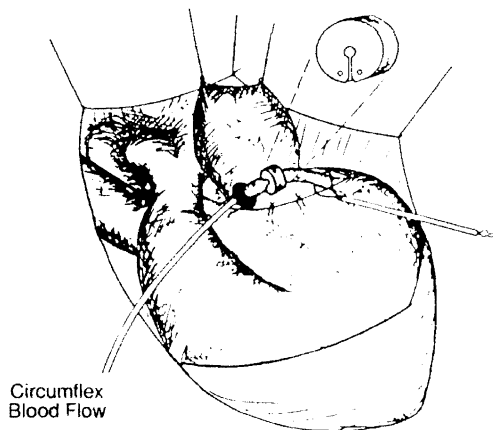


Figure 4 Technique for monitoring platelet aggregation in the partially obstructed left circumflex coronary artery of the dog. Electromagnetic flow probes measure blood flow. Partial obstruction of the coronary artery with a plastic Lexan cylinder results in episodic cyclical reductions in coronary blood flow that are due to platelet-dependent thrombus formation. Every 2–3 mm the thrombus must be mechanically shaken loose to restore blood. For detailed application of the Folts model, see Folts, Rowe (1974, 1988); and Folts et al. (1976, 1982)

EVALUATION

For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic [mm Hg]) is determined.

Protocols 1–4 The following parameters are determined to quantify stenosis-induced coronary thrombosis:

- Frequency of CFRs = cycle number per time
- Magnitude of CFRs = cycle area (mm^2)

Percent change in cycle number and cycle area after drug treatment is calculated compared to pre-treatment controls.

Statistical significance is assessed by the paired Student's *t*-test.

Protocol 5 The following parameters are determined to quantify electrically-induced coronary thrombosis:

- Occlusion time (min) = time to zero blood flow
- Thrombus size (mg) = wet weight of the thrombus immediately after removal.

Percent change in mean values for occlusion time and thrombus size in drug-treated groups is compared to the control group.

Statistical significance is assessed by the non-paired Student's *t*-test.

CRITICAL ASSESSMENT

Both, the stenosis (Folts) and the electrical (Romson/Lucchesi) model of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush and Patrick (1986) reviewed the role of the endothelium in arterial thrombosis and the effect of some inhibitors and mediators in the Folts model, e. g. thromboxane, prostacyclin, cyclooxygenase, serotonin, NO-donors and other vasodilators. The effect of an NO-donor could be reversed by the NO-scavenger oxyhemoglobin indication that indeed NO was responsible for the anti-thrombotic action (Just and Schönafinger 1991). Recent mechanisms of antithrombotic drug action that have been studied in either of the two coronary thrombosis models are the oral GP IIb/IIIa antagonist DMP 728 (Mousa et al. 1996); the LMWH enoxaparin (Leadley et al. 1998), which inhibited CFRs in contrast to unfractionated heparin; the thrombin inhibitors PEG-hirudin (Ruebsamen and Kirchengast 1998) and melagatran (Mehta et al. 1998); an anti-P-selectin antibody (Ikeda et al. 1999); and activated protein C (Jackson et al. 2000).

The clinical relevance of studies in the Folts model has been questioned because the model is very sensitive to antithrombotic compounds. However, the lack of a reversal of the effect by epinephrine or increase in degree of stenosis differentiates any new drug from aspirin. Electrical coronary thrombosis is less sensitive: e. g. aspirin has no effect, and with some drugs higher dose levels are required; however, in principle, most drug mechanisms act in both models if at all.

MODIFICATIONS OF THE METHOD

Romson et al. (1980b) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low-amperage electric current to the intimal surface of the artery.

Benedict et al. (1986) modified the electrical induction of thrombosis by use of two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped at 50% increase in flow velocity, and thrombosis then occurred spontaneously. The important role of serotonin was demonstrated by increases in coronary sinus serotonin levels just prior to occlusion.

Wartier et al. (1987) described a canine model of thrombin-induced coronary artery thrombosis, as well

as the effects of intracoronary streptokinase on regional myocardial blood flow, contractile function, and infarct size.

Al-Wathiqui et al. (1988) described the induction of cyclic flow reduction in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs.

The method of Folts thrombosis has also been applied to carotid arteries in monkeys. Collier et al. (1989) induced CFRs in carotid arteries of anesthetized cynomolgus monkeys and showed abolition by the GP IIb/IIIa antibody abciximab.

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B.4.2

Stenosis- and Mechanical Injury-Induced Arterial and Venous Thrombosis: Harbauer-Model

PURPOSE AND RATIONALE

Harbauer et al. (1984) and Harbauer and Allendorf (1988) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification, both arterial and venous thrombosis is produced in rabbits by stenosis of the carotid artery and the jugular vein with simultaneous mechanical damage of the endothelium. This activates platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed as detected by blood flow measurement. The dominant role of platelets in this model is shown by the inhibitory effect of an antiplatelet serum in both types of vessels (Just 1986). The test is used to evaluate the antithrombotic capacity of compounds in an *in vivo* model of arterial and venous thrombosis where thrombus formation is highly dependent on platelet activation.

PROCEDURE

Male Chinchilla rabbits weighing 3–4 kg receive the test compound or the vehicle (controls) by oral, in-

travenous or intraperitoneal administration. The first ligature (vein, preparation see below) is performed at the end of absorption (i.p. approx. 30 min, p.o. approx. 60 min, i.v. variable).

Sixty-five minutes before stenosis, the animals are sedated by intramuscular injection of 8 mg/kg xylazine (Rompun) and anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein. A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube. One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow in spite of vessel occlusion. Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as far central as possible). Blood flow (ml/min) is measured continuously. After reaching steady state (approx. 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching) and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approx. 1.5 min), the carotid artery is damaged by briefly squeezing it with forceps. Then a small plastic constricting cylinder 1.2 mm wide and 2 mm long is placed around the site of the endothelial damage.

In addition, the template bleeding time is measured at various time interval before and after drug treatment (depending on the route of administration) in the shaved inner ear using the Simplate device. Care is taken to select parts of the skin without larger vessels.

EVALUATION

Percent thrombus formation (= thrombosis incidence) is judged by determination of the number of occluded vessels (blood flow = 0).

Percent inhibition of thrombosis incidence is calculated in dosed groups as compared to vehicle controls. Thrombosis incidence is always 100% in vehicle controls.

Statistical significance is assessed by means of the Fisher exact test.

If initial values for blood flow do not significantly differ in dosage and control groups, the area below the blood flow curves is measured by planimetry in addition, and mean values in dosed groups are compared to controls by means of the unpaired Student's *t*-test. Mean values of occlusion times [min] in dosage and

control groups are calculated and compared by means of the t-test.

The maximal change in systolic and diastolic blood pressure during the period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. As an example, a reduction of systolic blood pressure by 30 mm Hg and of diastolic blood pressure by 20 mm Hg is quoted as a strong reduction in blood pressure.

CRITICAL ASSESSMENT OF THE METHOD

Two main factors of arterial thrombosis are essential in this model: high-grade stenosis and vessel wall damage. In the absence of either, no thrombus is found. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels thrombus formation is equally dependent on platelet function, as shown by antiplatelet serum. Therefore, the jugular vein thrombosis in this model differs from stasis-induced deep vein thrombosis with predominant fibrin formation. On the other hand, these occlusive thrombi are more stable than the pure platelet thrombi in the Folts model since carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs are effective: (i) antiplatelet drugs like ticlopidine, prostacyclin/iloprost, NO-donors (SNP, molsidomine) but not aspirin, thromboxane-synthase-inhibitors; (ii) anticoagulants like hirudin, high-dose heparin, warfarin; and (iii) streptokinase/t-PA (Bevilacqua et al. 1991, Just 1986). In contrast, drugs that only lower blood pressure—such as hydralazine, clonidine, and prazosin—have no effect on thrombus formation in this model.

MODIFICATIONS OF THE METHOD

Bevilacqua et al. (1991) performed the same model in rabbit carotid arteries but compared the procedure in one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH₂Cl, iloprost and t-PA inhibited carotid occlusion in this model but not aspirin.

Spokas and Wun (1992) produced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. The vascular wall was damaged by crushing with hemostat clamps. A segment of the vena cava was looped with two ligatures, 2.5 cm apart. At 2 h after ligation, the isolated venous sac was dissected and the clot removed for determination of dry weight.

Lyle et al. (1995) searched for an animal model mimicking the thrombotic reocclusion and restenosis occurring in several cases after successful coronary

angioplasty in man. The authors developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute ¹¹¹indium-labelled platelet deposition and thrombosis were assessed 4 hours after balloon injury in arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month). The effects of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

Thrombosis Induced by Cooling

Lindenblatt et al. (2005), Meng (1975), Meng and Seuter (1977) and Seuter et al. (1979) described a method to induce arterial thrombosis in rats by chilling of the carotid artery. Rats were anesthetized; the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove which was cooled to -15°C . The vessel was compressed by a weight of 200 g. In addition, a silver clip was fixed to the vessel distally from the injured area to produce a disturbed and slow blood flow. After 4 min, the proximal clamp was removed and the blood flow reestablished in the injured artery. In the rabbit, slightly different conditions were used: the chilling temperature was -12°C for a period of 5 min, and the compressing weight was 500 g. The wound was closed, and the animal was allowed to recover from anesthesia. Antithrombotic compounds were administered in various doses at different time intervals before surgery. After 4 hours, the animals received heparin and were reanesthetized. The lesioned carotid artery was removed and thrombus wet-weight was immediately measured.

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B.4.3

Electrical-Induced Thrombosis

PURPOSE AND RATIONALE

The use of electrical current to induce thrombosis in hamster and dog has been described in the early 1950s by Lutz et al. (1951) and Sawyer et al. (1953a, b). In general, two different approaches exist. One method produces electrical damage by means of two externally applied hook-like electrodes (Hladovec 1973, Philp et al. 1978). The other method uses a needle electrode that is advanced through the walls of the blood vessels and positioned in their lumen; the second electrode is placed into a subcutaneous site completing the circuit (Salazar 1961, Romson et al. 1980, Benedict et al. 1986).

PROCEDURE

Anaesthetized rats weighing 200–300 g are intubated, and a femoral artery is cannulated for administration of drugs. One carotid artery is isolated from surrounding tissues over a distance of 10–15 mm.

A pair of rigid stainless-steel wire hook-like electrodes with a distance of 4 mm are adjusted to the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flowmeter (Transonic, Ithaca NY, USA); the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimula-

tor (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between onset of the electrical current and the time at which blood flow decreases under 0.3 ml/min
- Patency of the blood vessel over 30 min.

CRITICAL ASSESSMENT OF THE METHOD

The electrical-induced thrombus is composed of densely packed platelets with some red cells. Moreover, the electrical injury causes extensive damage to intimal and subintimal layers. The endothelium is completely destroyed, and this damage extends to subendothelial structures including smooth muscle cells. The deep damage could reduce the possibility of discrimination between drugs on the basis of their antithrombotic activity. However, Philp et al. (1978) could show that unfractionated heparin completely blocked thrombus formation, whereas other antiplatelet agents displayed differentiated antithrombotic action. He concluded that this relatively simple model of arterial thrombosis might prove a useful screening test for drugs with antithrombotic potential.

MODIFICATIONS OF THE METHOD

The technique described by Salazar et al. (1961) uses a stainless steel electrode that is inserted into a coronary artery in the dog and that delivers anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control, which complicates the method. The technique was modified by Romson et al. (1980). They placed the electrode directly into the coronary artery of open-chest anaesthetized dogs.

Rote et al. (1993, 1994) used a carotid thrombosis model in dogs. A calibrated electromagnetic flow meter was placed on each common carotid artery proximal to both the point of insertion of an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern decreased by 25% without altering the mean blood flow. Electrolytic injury to the intimal surface was accomplished with the use of an intravascular electrode composed of a Teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V nickel-cadmium battery. The cathode was connected to a subcutaneous site. Injury was initiated in the right carotid artery by application of a 150 μ A continuous pulse anodal direct current to the intimal sur-

face of the vessel for a maximum duration of 3 hours or for 30 min beyond the time of complete vessel occlusion, as determined by the blood flow recording. Upon completion of the study on the right carotid, the procedure for induction of vessel wall injury was repeated on the left carotid artery after administration of the test drug.

Benedict et al. (1986) introduced a procedure in which anodal current is discontinued when mean distal coronary flow velocity increased by approximately 50%, reflecting disruption of normal flow by the growing thrombus. Occlusive thrombosis occurred within 1 hour after stopping the electrical current. It was observed that the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (1996). A 10-min anodal electrical stimulation of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle-treated ferrets within 41 ± 3 min, with an average weight of 8 ± 1 mg. Thrombus weight was reduced by aspirin or a thromboxane receptor antagonist.

Guarini (1996) produced a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

Sturgeon et al. (2006) adapted the Folts and the electric methods of arterial thrombosis in small animals. Mousa et al. (1999) used the same animal pre- and post-treatment by using left and right arterial sides.

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B.4.4

FeCl₃-Induced Thrombosis

PURPOSE AND RATIONALE

A variety of chemical agents has been used to induce thrombosis in animals. Topical FeCl₃ was described by Reimann-Hunziger (1944) and recently by Wang et al. (2006) as thrombogenic stimulus in veins. Kurz et al. (1990) showed that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This model is used as a simple and reproducible test for evaluation of antithrombotic (Broersma et al. 1991) and profibrinolytic test compounds (van Giezen et al. 1997).

PROCEDURE

Rats weighing between 250 and 300 g are anaesthetized with Inactin (100 mg/kg), and a polyethylene catheter (PE-205) is inserted into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood samples and measurement of arterial blood pressure and in the jugular vein for administration of test agents. The right carotid artery is isolated and an ultrasonic Doppler flowprobe (probe 1RB, Transonic, Ithaca NY, USA) is placed on the vessel to measure blood flow. A small piece of Parafilm “M” (American Can Co, Greenwich,

CT) is placed under the vessel to isolate it from surrounding tissues throughout the experiment.

The test agent is administered by gavage or as an intravenous injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of filter paper (2 × 5 mm), saturated with 25% FeCl₃ solution, to the carotid artery. The paper is allowed to remain on the vessel 10 min before removal. The experiment is continued for 60 min after the induction of thrombosis. At that time, the thrombus is removed and weighed.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between FeCl₃ application and the time at which blood flow decreases under 0.3 ml/min
- Thrombus weight after blotting the thrombus on filter paper

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B.4.5

Thrombin-Induced Clot Formation in Canine Coronary Artery

PURPOSE AND RATIONALE

A canine model of thrombin-induced clot formation was developed by Gold et al. (1984) in which localized coronary thrombosis was produced in the LAD. This is a variation of the technique described by Collen et al. (1983) who used radioactively labeled fibrinogen to monitor the occurrence and extent of thrombolysis of rabbit jugular veins clots. The vessel was intentionally de-endothelialized by external compression with blunt

forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) also was injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 min later, and total occlusion was confirmed by selective coronary angiography. This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 hours, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.

PROCEDURE

In the initial study described by Gold et al. (1984), recombinant t-PA was characterized for its ability to lyse 2-hour-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25 µg/kg/min, i.v, and resulted in reperfusion times of 40, 31, and 13 min, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis. Furthermore, it is possible to study the effect of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and a₂-antiplasmin, as well as to assess myocardial infarct size. For example, Kopia et al. (1988) demonstrated that streptokinase (SK) elicited dose-dependent thrombolysis in this model.

Subsequently, Gold et al. (1986 and 1988) modified the model to study not only reperfusion but also acute reocclusion. Clinically, reocclusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45% of patients (Goldberg et al. 1985). Thus, an animal model of coronary reperfusion and reocclusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute reocclusion.

Thrombin-Induced Rabbit Femoral Artery Thrombosis: Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride, and fresh blood via a side branch (Shebuski et al. 1988).

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with the

jaws of forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow (FABF). The superficial epigastric artery is cannulated for induction of the thrombus and subsequent infusion of thrombolytic agents. Localized thrombi distal to the lateral circumflex artery with snares approximately 1 cm apart are induced by the sequential injection of thrombin, CaCl₂ (1.25 mmol), and a volume of blood sufficient to distend the artery. After 30 min, the snares are released and FABF is monitored for 30 min to confirm total obstruction of flow by the thrombus.

EVALUATION

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA occurs with subsequent reocclusion. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al. (1983) and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute reocclusion (Yasuda et al., 1988). These actions *in vivo* were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

REFERENCES AND FURTHER READING

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B.4.6

Laser-Induced Thrombosis

PURPOSE AND RATIONALE

Thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser beams. The test can be performed in normal or pretreated (induction of arteriosclerosis or adjuvant arthritis) animals. The mediators for thrombus formation in this method are platelet adhesion to the injured endothelial vessel wall on one hand and ADP-induced platelet aggregation on the other. Most probably, ADP is primarily released by laser beam-lysed erythrocytes, due to the fact that erythrocyte hemoglobin exerts strong adsorbability to frequencies emitted by laser beams. There is a further, secondary, aggregation stimulus following the release reaction induced by the platelets themselves.

PROCEDURE

Apparatus

- 4 W argon laser (Spectra Physics, Darmstadt, FRG); wave length: 514.5 nm; energy below the objective: 15 mW; duration of exposure: 1/30 or 1/15 sec
- Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
- Video camera (Sony, Tricon tube)
- Recorder (Sony, U-matic 3/4")
- Videoanalyzer and correlator to determine blood flow velocity

In Vivo Experiment

Male Sprague Dawley or spontaneously hypertensive stroke-prone Wistar or Lewis rats with adjuvant induced arthritis weighing 150–300 g or New Zealand rabbits with arteriosclerosis induced by cholesterol feeding for 3 months are used. The animals receive the test compound by oral, intravenous, intraperitoneal, or subcutaneous administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anaesthetized

by intraperitoneal administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60 or 90 min post dosing. Investigations are performed in arterioles or venules of $13 \pm 1 \mu\text{m}$ in diameter of the fat-free ileocaecal portion of the mesentery. During the test procedure, the mesenterium is superfused with physiological saline solution or degassed paraffin liquid (37°C). The ray of the argon laser is led into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injuries is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 sec. The number of injuries necessary to induce a defined thrombus is determined. All thrombi formed during the observation period with a minimum length of $13 \mu\text{m}$ or an area of at least $25 \mu\text{m}^2$ are evaluated. All measuring procedures are photographed by a video system.

Standard compounds:

- acetylsalicylic acid (10 mg/kg, per os)
- pentoxifylline (10 mg/kg, per os)

For detailed description and evaluation of various agents and mechanisms see the following references: Arfors et al. (1968), Herrmann (1983), Seiffge and Kremer (1984 and 1986), Seiffge and Weithmann (1987), and Weichert et al. (1983).

EVALUATION

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated.

REFERENCES AND FURTHER READING

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B.4.7

Photochemical-Induced Thrombosis

PURPOSE AND RATIONALE

In 1977, Rosenblum and El-Sabban reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein. They found that in contrast to heparin, both aspirin and indomethacin prolonged the time to first platelet aggregate. Herrmann (1983) provided a detailed study in which he showed that scavengers of singlet oxygen, not of hydroxyl radicals, inhibited platelet aggregation induced by the photochemical reaction. He postulated that by exciting the intravascularly administered fluorescein, singlet oxygen damages endothelial cells, which subsequently leads to platelet adhesion and aggregation.

PROCEDURE

Studies are performed in mesenteric arteries of 15–30 μm diameter in anesthetized rats. After intravenous injection of fluorescein isothiocyanate-dextran 70 (FITC-dextran, Sigma, 10%, 0.3 ml), the FITC-dextran in arterioles is exposed to ultraviolet light (wavelength of excitation 490 nm, wavelength of emission 510 nm).

EVALUATION

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to other thrombosis induction methods, photochemically induced thrombosis can be easily used in smaller animals. Thrombi are composed primarily of platelets; however, the primary target of the photochemical insult is the endothelial cells by means of oxygen radical damage.

MODIFICATIONS OF THE METHOD

Matsuno et al. (1991) report a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (rose Bengal, 10 mg/kg i.v.) and transillumination with a filtered xenon lamp (wave length: 540 nm). Blood flow is monitored by a pulsed Doppler flow meter. Occlusion is achieved after approximately 5–6 min. Pre-treatment with heparin dose-dependently prolongs the time required to interrupt the blood flow. The model also enables one to study thrombolytic mechanisms, which had been evaluated with t-PA. A comparative

data for hirudin in various models was carried out by Just et al. (1991).

REFERENCES AND FURTHER READING

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B.4.8

Foreign-Surface-Induced Thrombosis

The presence of foreign materials in the circulation produces activation of the coagulation and the platelet system. Various prothrombotic surfaces have been used to develop experimental animal models. In contrast to many other thrombosis models, the thrombosis induced by foreign surfaces does not presuppose endothelial damage.

B.4.8.1

Wire Coil-Induced Thrombosis

PURPOSE AND RATIONALE

A classical method to produce thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (1951) in aorta of dogs and was further modified to be used in arterial coronary vessels of opened-chest dogs. The use in venous vessels was described by Kumada et al. (1980).

The formation of thrombotic material around the coil is reproducible and can be easily standardized to study pharmacological agents (Just and Schönafinger 1991, Mellott et al. 1993, Rübsamen and Hornberger 1996).

Venous thrombosis is produced in rats by insertion of a stainless steel wire coil into the inferior caval vein. Platelets as well as plasmatic coagulation are activated on the wire coil. Thrombus formation onto the wire is quantitated by measuring the protein content of the thrombotic material isolated. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min, followed by a steady state

between thrombus formation and endogenous thrombolysis leading to a constant protein content of thrombi between 1 and up to 48 hours following implantation of the wire coil. Thrombosis incidence in untreated control animals in this model is 100%. The test is used to evaluate antithrombotic and thrombolytic properties of compounds in an *in vivo* model of venous thrombosis in rats.

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. At the end of absorption (i.v. 1 min, i.p. 30 min, p.o. 60 min), the animals are anesthetized by intraperitoneal injection of 1.3 g/kg urethane. Through a mid-line incision the caudal caval vein is exposed and a stainless steel wire coil (a dental pate carrier, Zipperer size 40(st), Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting of the wire toward the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured and the animal is placed on its back on a heating pad (37°C). The wound is reopened after 2 hours; the wire coil is carefully removed together with the thrombus on it and rinsed with 0.9% saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution (2% Na₂CO₃ in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 µl aliquots by the colorimetric method of Lowry. See figure below (Fig. 5).

Thrombolysis

In addition to the described preparation, for continuous infusion of a thrombolytic test solution a polyethylene catheter is inserted in the jugular vein. One and a half hours after implantation of the wire coil, the test compound or the vehicle (controls) is infused for up to 2.5 hours. The wire coil is then removed and the protein content of thrombi is determined (see above). Bernat et al. (1986) demonstrated the fibrinolytic activity of urokinase and streptokinase-human plasminogen complex in this model.

EVALUATION

Thrombosis incidence (= number of animals with thrombi in dosage groups as compared to vehicle controls) is assessed.

The mean protein content (mg) of the thrombotic material in dosage groups and vehicle controls is deter-

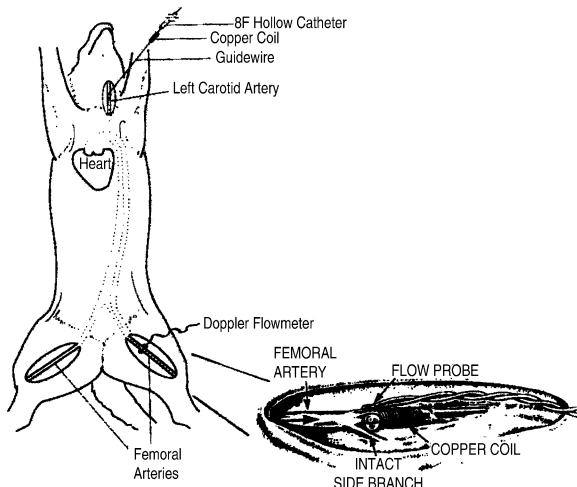


Figure 5 Schematic diagram of the canine femoral artery copper coil model of thrombolysis. A thrombogenic copper coil is advanced to either femoral artery via the left carotid artery. By virtue of the favorable anatomical angles of attachment, a hollow polyurethane catheter advanced down the left carotid artery nearly always enters the descending aorta, and with further advancement, into either femoral artery without fluoroscopic guidance. A flexible, Teflon-coated guidewire is then inserted through the hollow catheter and the latter is removed. A copper coil is then slipped over the guidewire and advanced to the femoral artery (see inset). Femoral artery flow velocity is measured directly and continuously with a Doppler flow probe placed just proximal to the thrombogenic coil and distal to a prominent sidebranch, which is left patent to dissipate any dead space between the coil and the next proximal sidebranch. Femoral artery blood flow declines progressively to total occlusion over the next 10–12 mm after coil insertion

mined. Percent change in protein content is calculated in dosage groups as compared to controls.

Statistical significance is assessed by means of the unpaired Student's *t*-test.

REFERENCES AND FURTHER READING

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B.4.8.2

Eversion Graft-Induced Thrombosis

PURPOSE AND RATIONALE

The eversion graft model for producing thrombosis in the rabbit artery was first described by Hergrueter et al. (1988) and later modified by Jang et al. (1989, 1990) and Gold et al. (1991). A 4- to 6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted and then reimplanted into the vessel by end-to-end anastomoses. After restoration of the blood flow, a platelet-rich occlusive thrombus forms rapidly, leading to complete occlusion of the vessel. This model mimics a deep arterial injury since the adventitial surface is a non-endothelial tissue containing tissue factor and collagen. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction often occurring in the inverted femoral segments.

PROCEDURE

In anaesthetized New Zealand White rabbits, the right carotid artery is exposed. After double ligation, a 3-mm segment of the artery is excised, everted and immersed in prewarmed (37°C) isotonic saline. Thereafter, the right femoral artery is exposed and occluded by means of a double-occluder (2-cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures with 9–0 nylon (Prolene, Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650, Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flowmeter (Model T106, Transonic, Ithaca, NY, USA). The flow probe is positioned 2 cm distal from the graft. After a stabilization period of 15 min, the test substance is given intravenously through the catheterized right jugular vein. Ten minutes after substance administration, the vessel clamps are released and the blood flow is monitored by the flowmeter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before substance administration), 10 min, 60 min and 120 min after substance administration.

EVALUATION

- Time until occlusion (time after restoring of vessel

blood flow until occlusion of the vessel, indicated by a flow less than 3.0 ml/min)

- Patency (time during which perfusion of graft is measured related to an observation period of 120 min after administration of test compounds).

Statistical Analysis

Time until occlusion and patency are expressed as median and the interquartile range/2 (IQR/2). Significant differences ($p < 0.05$) are calculated by the non-parametric Kruskal-Wallis test.

CRITICAL ASSESSMENT OF THE METHOD

The eversion graft is very thrombogenic, although technically difficult and time consuming. The deep occlusive thrombi can be prevented only by intraarterially administered thrombolytics or aggressive antithrombotic treatments such as recombinant hirudin at high dosages, or PEG-hirudin. The adventitial surface is a non-endothelial tissue containing tissue factor and collagen. Thus, both the coagulation system and blood platelets are activated.

MODIFICATIONS OF THE METHOD

Gold et al. (1991) modified the model to be used in thoracotomized dogs in partial obstructed left circumflex coronary arteries. The combination of reduced blood flow due to the constrictor, along with an abnormal non-endothelial surface, produces total thrombotic occlusion within 5 min.

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B.4.8.3

Arteriovenous Shunt Thrombosis

PURPOSE AND RATIONALE

A method for the direct observation of extracorporeal thrombus formation was introduced by Rowntree and Shionoya (1927) and extensively utilized by oth-

ers (Rushkin et al. 2003; Tang et al. 2003). These first studies could provide evidence that anticoagulants like heparin and hirudin do inhibit thrombus development in arteriovenous shunts. Since today, the A-V-shunt thrombosis models have been often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (Knabb et al. 1992), rats (Hara et al. 1995), pigs (Scott et al. 1994), dogs and cats (Best et al. 1938), and non-human primates (Yokoyama et al. 1995).

PROCEDURE

Rats are anaesthetized and fixed in supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are connected with a 2-cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of the test compound, the clamps that are occluding the A-V-shunt are opened.

The measurement of the patency of the shunt is performed indirectly with a NiCrNi-thermocouple, which is fixed distal to the glass capillary. If blood is flowing, the temperature rises from room temperature to body temperature. In contrast, decreases of temperature indicate the formation of an occluding thrombus. The temperature is measured continuously over 30 min after opening of the shunt.

CRITICAL ASSESSMENT OF THE METHOD

It has been shown by Best et al. (1938) that the thrombi formed in the AV-shunt are to a greater part white arterial thrombi. This might be due to the high pressure and shear rate inside the shunts; in those cases, the thrombi tend to be more arterial in character (Chi et al. 1999).

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B.4.8.4

Thread-Induced Venous Thrombosis

PURPOSE AND RATIONALE

Compared to the arterial system, it seems to be more difficult to develop a thrombosis model in venous blood vessels with respect to reproducibility and variability (Chi et al. 1999). Complete stasis together with a thrombogenic stimulus (Wessler-type) is used by numerous investigators to evaluate the effect of compounds on venous thrombosis. Hollenbach et al. (1994) developed a rabbit model of venous thrombosis by inducing cotton threads into the abdominal vena cava of rabbits. The cotton threads serve as a thrombogenic surface, and a thrombus forms around it, growing to a maximum mass after 2–3 hours. The prolonged non-occlusive character of thrombogenesis in this model focuses on progression of thrombus formation rather than initiation. Therefore, the conditions more closely resemble pathophysiology in humans because blood continues to flow throughout the experiment (Chi et al. 1999).

PROCEDURE

Rabbits weighing between 2.5 and 3.5 kg are anaesthetized with isoflurane inhalation anesthesia, and a polyethylene catheter is inserted into the left carotid artery. A polyethylene tube (PE 240, inner diameter 1.67 mm) of 14 cm length is filled with isotonic saline, and a copper wire with 5 fixed cotton threads (length 6 cm) is inserted into the tube (after determination of the net weight of the cotton threads). A laparotomy is performed and the vena cava and iliac vein are dissected free from surrounded tissue. The test agent is

administered by a rabbit intragastric tube 60 min (depending on the *ex vivo* study) prior to initiation of thrombus formation. Blood samples will be measured at 60, 90, 120, 150 and 210 min after oral administration of the test compound.

Thrombus formation is induced by the inserting the thrombosis catheter into the caval vein via the iliac vein (7 cm). Then the copper wire is pushed forward 3 cm to liberate the cotton threads into the vessel lumen. One hundred fifty minutes after thrombus initiation, the caval segment containing the cotton threads and the developed thrombus will be removed, longitudinally opened and the content blotted on filter paper. After weighing the cotton thread with the thrombus, the net thread weight will be subtracted to determine the corrected thrombus weight.

EVALUATION

- Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net weight of the cotton thread
- Mean arterial blood pressure
- APTT, HepTest, antiFIIa and antiFXa activity.

CRITICAL ASSESSMENT OF THE METHOD

The composition of the cotton-threaded thrombus shows a composition of fibrin together with tightly aggregated and distorted erythrocytes, thus being in accordance with human deep-vein thrombosis structure. Non-occlusive thrombus formation has been successfully inhibited by heparins, prothrombinase complex inhibitors and thrombin inhibitors (Hollenbach et al. 1994, 1995).

MODIFICATIONS OF THE METHOD

In addition to the originally described method, it is possible to measure blood flow by means of an ultrasonic flow probe, attached distally to the position of the cotton threads on the vein.

REFERENCES AND FURTHER READING

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B.4.8.5**Thrombus Formation on Superfused Tendon****PURPOSE AND RATIONALE**

In all models that include vessel wall damage, blood gets in contact with adhesive proteins of the subendothelial matrix, i.e. von Willebrand factor, collagens, fibronectin, laminin and others. Gryglewski et al. (1978) described an *in vivo* method where blood of an unanesthetized animal is in contact *ex vivo* with a foreign surface consisting mainly of collagen. The foreign surface is produced out of the tendon of another animal species. After superfusion of the tendon, blood is recirculated to the unanesthetized animal. The method aims at the quantitation of the antiplatelet potency of drugs based on the formation of platelet thrombi onto the surface of the tendons or of aortic strips from atherosclerotic rabbits.

PROCEDURE

Blood was withdrawn from the carotid artery of anesthetized and heparinized cats by a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37°C), blood was separated into 2 streams, each flowing at a speed of 3 ml/min superfusing in parallel 2 twin strips of the central part of longitudinally cut rabbit Achilles tendon (30 × 3 mm). The blood superfusing the strips dripped into collectors and by its gravity was returned to the venous system of the animals through the left jugular vein. The tissue strips were freely suspended in air and the upper end was tied to an auxotonic lever of a smooth muscle/heart Harvard transducer, while the lower end was loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips were successively covered with clots changing the weight of the strips. The weight changes were continuously recorded. After a control period of 30 min, the formed thrombi were gently removed and fixed in formalin for histological examination. Then, the strips were superfused with Tyrode solution and the animals injected with the antithrombotic drug. After 10 min, blood superfusion was renewed for another 30 min.

EVALUATION

The ratio of an increase in weight of the strips after the drug treatment to the increase in weight before drug treatment was considered as an index of anti-aggregatory activity.

REFERENCES AND FURTHER READING

Gryglewski RJ, Korbut R, Ocetkiewicz A, Stachura J (1978) *In vivo* method for quantitation of anti-platelet po-

tenacy of drugs. Naunyn-Schmiedeberg's Arch Pharmacol 302:25–30

B.4.9**Stasis-Induced Thrombosis (Wessler Model)****PURPOSE AND RATIONALE**

The "Wessler model" is a classical method of inducing venous thrombosis in animals. Wessler (1952, 1953, 1955a & b, 1957) and Wessler et al. (1959) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after the injection of the procoagulatory stimulus into the circulation. Within a few minutes after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (1985) summarized a variety of substances that can be used as pro-coagulatory stimuli. Aronson and Thomas (1985) found an inverse correlation between the duration of stasis and the amount of the hypercoagulating agents to produce the clot.

PROCEDURE

Anaesthetized rabbits are fixed in supine position on a temperature-controlled (37°C) heating table. Following cannulation of both carotid arteries (the left in cranial direction) and the right femoral vein, segments of 2 cm length of the two external jugular veins are exposed and isolated between two loose sutures. Then, 0.3 ml/kg calcium thromboplastin (SIGMA, Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 sec followed by injection of 0.5 ml physiological saline within 15 sec; 45 sec later, both jugular vein segments are occluded by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 sec before end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by a longitudinal incision.

EVALUATION

The size of the clots is assessed using a score system: (0: blood only; 1: very small clot piece[s], filling out at most 1/4 of the vessel; 2: larger clot piece[s], filling out at most 1/2 of the vessel; 3: very large clot[s], filling out at most 3/4 of the vessel; 4: one large clot, filling out the whole vessel). The scores of the left and the right jugular vein are added, forming the thrombus size value of one animal. Additionally, the thrombus weight is measured after blotting the thrombus on filter paper.

Thrombus score is expressed as median (minimum–maximum). Thrombus weight is given as mean \pm SEM. For the statistical evaluation of the antithrombotic effect, the nonparametric U-Test of Mann and Whitney (thrombus score) or Student's *t*-test for unpaired samples (thrombus weight) is used. Significance is expressed as $p < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

Breddin (1989) described the Wessler model because of its static character as the retransformation of an *in vitro* experiment into a very artificial test situation. One of the major drawbacks is the relative independence of platelet function and hemodynamic changes that largely influence thrombus formation *in vivo*. However, the model has been shown to be very useful for evaluation of the antithrombotic effect of compounds like heparin and hirudin.

MODIFICATIONS OF THE METHOD

There are a number of different procoagulant agents that had been used to induce thrombosis in this model, such as human serum, Russel viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates and factor X_a (Aronson and Thomas 1985, Fareed et al. 1985). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring the specific radioactivity in the clot.

The general drawback of the Wessler model is the static nature of the venous thrombus development. To overcome this problem some investigators have developed more dynamic models with reperfusion of the occluded vessel segments after clot development. Depending on the time of test compound administration (pre- or post-thrombus initiation), the effect on thrombus growth and fibrinolysis can be evaluated. Levi et al. (1992) have used this model to assess the effects of a murine monoclonal anti-human PAI-1 antibody, and Biemond et al. (1996) compared the effect of thrombin-and factor X_a-inhibitors with a low molecular weight heparin.

Venous reperfusion model: New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml atropine, 1.0 mg/kg diazepam, and 0.3 ml Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 ml fentanyl). Further anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposition through an incision in the neck. The jugular vein is dissected free from tissue, and small side branches are ligated over a distance of 2 cm. The vein is clamped

proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with ¹³¹I-radiolabeled fibrinogen (final radioactivity, approximately 25 mCi/ml). Then, 150 μ l of this blood is aspirated in a 1-ml syringe containing 25 μ l thrombin (3.75 IU) and 45 μ l 0.25 mol CaCl₂, and 200 μ l of the clotting blood is immediately injected into the isolated segment. The vessel clamps are removed 30 min after clot injection, and blood flow is restored. ¹²⁵I-radio-labeled fibrinogen (approximately 5 μ Ci) is injected through the cannula in the carotid artery, (in case of the fibrinolysis studies immediately followed by 0.5 mg/kg recombinant tissue-type plasminogen activator). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measurement of the remaining ¹³¹I-fibrinogen in the clot and compared with the initial clot radioactivity. The comparison between blood and thrombus ¹²⁵I-radioactivity reveals the extent of thrombus growth (blood volume accreted to the blood). The thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as percentage of the initial thrombus volume. Statistics is performed as variance analysis and the Newman-Keuls test. Statistical significance is expressed at the level of $p < 0.05$.

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B.4.10

Disseminated Intravascular Coagulation (DIC) Model

PURPOSE AND RATIONALE

DIC is another model that is also used widely in rats and mice. It is a model of systemic thrombosis or disseminated intravascular coagulation (DIC), which is induced by tissue factor, endotoxin (lipopolysaccharide), or FXa (Herbert et al. 1996, Yamazaki et al. 1994, Sato et al. 1998). After systemic administration of the thrombogenic stimulus, this model can be performed with or without mechanical vena caval stasis. When stasis is used, the major parameter is the thrombus mass, but when stasis is not used, the readouts are fibrin degradation products, fibrinogen, platelet count, PT, and APTT, among others. As shown by the many and varied parameters, when used without stenosis, the post-experimental analysis can be time-consuming and technically demanding. Although rodents are useful as a primary efficacy model, limitations such as the ability to withdraw multiple blood samples over the course of the experiment and the difference in activity of at least some FXa inhibitors in human compared to rat plasma *in vitro* require that compounds be characterized further in more advanced *in vivo* models of thrombosis.

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B.4.11

Microvascular Thrombosis in Trauma Models

PURPOSE AND RATIONALE

Successful replantation of amputated extremities is dependent in large degree on maintaining the microcirculation. A number of models have been developed in

which blood vessels are subjected to crush injury with or without vascular avulsion and subsequent anastomosis (Fu et al. 1997, Korompilias et al. 1997, Stockmans et al. 1997). In the model of Stockmans (1997), both femoral veins are dissected from the surrounding tissue. A trauma clamp, which has been adjusted to produce a pressure of 1,500 g/mm², is positioned parallel to the long axis of the vein. The anterior wall of the vessel is grasped between the walls of the trauma clamp and the two endothelial surfaces are rubbed together for a period of 30 sec as the clamp is rotated. Formation and dissolution of platelet-rich mural thrombi are monitored over a period of 35 min by transillumination of the vessel. By using both femoral veins, the effect of drug therapy can be compared to control in the same animal, minimizing intra-animal variations.

The models of Korompilias et al. (1997) and Fu et al. (1997) examine the formation of arterial thrombosis in rats and rabbits, respectively. In these models, either the rat femoral artery or the rabbit central ear artery is subjected to a standardized crush injury. The vessels are subsequently divided at the midpoint of the crushed area and then anastomosed. Vessel patency is evaluated by milking the vessel at various time points post-anastomosis. These models have been used to demonstrate the effectiveness of topical administration of LMWH in preventing thrombotic occlusion of the vessels. Such models, while effectively mimicking the clinical situation, are limited by the necessity of a high degree of surgical skill to effectively anastomose the crushed arteries.

REFERENCES AND FURTHER READING

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B.4.12

Cardiopulmonary Bypass Models

PURPOSE AND RATIONALE

Cardiopulmonary bypass (CPB) models have been described in baboons (Van Wyk et al. 1998), swine (De-

wanjee et al. 1996) and dogs (Henny et al. 1985). In each model, the variables that can affect the hemostatic system—such as anesthesia, shear stresses caused by the CPB pumps and the exposure of plasma components and blood cells to foreign surfaces (catheters, oxygenators, etc)—are comparable to that observed with human patients. With these models, it is possible to examine the potential usefulness of novel anticoagulants in preventing thrombosis under relatively harsh conditions where both coagulation and platelet function are altered. The effectiveness of direct thrombin inhibitors (Van Wyk et al. 1998), LMWHs (Murray 1985) and heparinoids (Henny et al. 1985) has been compared to standard heparin. Endpoints have included the measurement of plasmatic anticoagulant levels, the histological determination of microthrombi deposition in various organs, the formation of blood clots in the components of the extracorporeal circuit and the deposition of radiolabeled platelets in various organs and on the components of the extracorporeal circuit. These models, therefore, can be used to assess the antithrombotic potential of new agents for use in CPB surgery and also to assess the biocompatibility of components used to maintain extracorporeal circulation. For detailed protocols and evaluations see Callas et al. (1995), Carrie et al. (1994), Fu et al. (1997), Korompilias et al. (1997), Meuleman et al. (1991), Millet et al. (1994), Stockmans et al. (1997), Vlasuk et al. (1991), Walenga et al. (1987), and Wessler et al. (1959).

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B.4.13

Extracorporeal Thrombosis Models

PURPOSE AND RATIONALE

These models employ passing blood over a section of damaged vessel (or other selected substrates) and recording the thrombus accumulation on the damaged vessel histologically or by scintigraphic detection of radiolabeled platelets or fibrin (Badimon and Badimon 1989). This model is interesting because the results can be directly compared to the *in vivo* deep arterial injury model (Wysokinski et al. 1996) results and to results from a similar extracorporeal model used in humans (Dangas et al. 1998; Ørvm et al. 1995). Dangas et al. (1998) used this model to characterize the antithrombotic efficacy of abciximab, a monoclonal antibody-based platelet glycoprotein IIb/IIIa inhibitor, after administration to patients undergoing percutaneous coronary intervention. They demonstrated that abciximab reduces both the platelet and fibrin components of the thrombus, thereby providing further insight into the unique long-term effectiveness of short-term administration of this drug. Ørvm et al. (1995) also used this model in humans to evaluate the antithrombotic efficacy of rTAP, but instead of evaluating the compound after administration of rTAP to the

patient, the drug was mixed with the blood immediately as it flowed into the extracorporeal circuit prior to flowing over the thrombogenic surface. By changing the thrombogenic surface, they were able to determine that rTAP was more effective at inhibiting thrombus formation on a tissue-factor coated surface compared to a collagen-coated surface. These results suggest that optimal antithrombotic efficacy requires an antiplatelet approach along with an anticoagulant. Although this model does not completely represent pathological intravascular thrombus formation, the use of this “human model” of thrombosis may be very useful in developing new drugs because it directly evaluates the *ex vivo* antithrombotic effect of a drug in flowing human blood.

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B.4.14

Experimental Thrombocytopenia or Leucocytopenia

PURPOSE AND RATIONALE

Intravenous administration of collagen, arachidonic acid, ADP, platelet activating factor (PAF) or thrombin activates thrombocytes leading to a maximal thrombocytopenia within a few minutes. The effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravascular aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of agonist, this experimentally induced reduction of the number of circulating platelets is reversible within 60 min after induction. Following administration of PAF, a leucocytopenia is induced in addition. The assay is used to test the inhibitory capacity of drugs against thrombocytopenia or leucocytopenia as a consequence of *in vivo* platelet or leukocyte stimulation.

PROCEDURE

Male guinea pigs (Pirbright White) weighing 300–600 g, or male NMRI mice (25–36 g), or Chinchilla rabbits of either sex weighing 2–3 kg are used. Animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (p.o. 60 min, i.p. 30 min, i.v. variable), the marginal vein of the ear of rabbits is cannulated and the thrombocytopenia-inducing substances collagen or arachidonic acid are injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun (i.m.) and placed on an electrically warmed table at 37°C. The carotid artery is cannulated for blood withdrawal and the jugular vein is cannulated to administer the thrombocytopenia-inducing substances collagen+adrenaline (injection of the mixture of both within 10 sec) or PAF or thrombin. In mice, collagen + adrenaline are injected into a tail vein.

Approximately 50–100 µl blood is collected into potassium-EDTA-coated tubes at times –1, 1 and 2 min (guinea pigs and mice) or 5, 10 and 15 min (rabbits) following the injection of the inducer. The number of platelets and leukocytes is determined within 1 hour after withdrawal in 10 µl samples of whole blood using a microcellcounter suitable for blood of various animal species.

EVALUATION

The percentage of thrombocytes (or leukocytes) is determined in vehicle control and dosage groups at the different times following injection of the inducer relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are taken as 100%.

Percent inhibition of thrombocytopenia (or leucocytopenia) is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The method of collagen+epinephrine-induced thrombocytopenia is presently widely used to study the phenotype of mice knocked out for a specific gene with suspected role in hemostasis/thrombosis. A recent example is the *Gas 6*^{–/–} mouse (Angelillo-Scherrer et al. 2001) and mice lacking the gene for the G protein G(z) (Yang et al. 2000). The advantage of the method for this purpose is the simple experimental procedure

and the small volume of blood necessary. In general, application of the method in small animals (mice, hamsters) needs only small amounts of drug substance. The model is a useful first step of *in vivo* antithrombotic efficacy of antiplatelet drugs.

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B.4.15

Collagenase-Induced Thrombocytopenia

PURPOSE AND RATIONALE

Intravenous administration of the proteolytic enzyme collagenase leads to formation of endothelial gaps and to exposure of deeper layers of the vessel wall. This vascular endothelial injury is mainly involved in triggering thrombus formation by activation of platelets through contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. The model is used to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats as an alternative to the model described before.

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g are used. The animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), rats are anesthetized with pentobarbital sodium (i.p.). One carotid artery is cannulated for blood withdrawal and one jugular vein is cannulated for inducer injection. The animals receive an intravenous injection of heparin and 20 min later, approximately 100 µl blood is collected (initial value). Ten minutes later, the thrombocytopenia-inducing substance collagenase is administered intravenously.

At times 5, 10, 20 and 30 min following the injection of collagenase, samples of approximately 100 µl blood are collected into potassium-EDTA-coated tubes. The number of platelets is determined in 10 µl samples of whole blood within 1 hour after

blood withdrawal, using a microcellcounter. See Völk and Dierichs (1986) for details.

EVALUATION

The percentage of platelets is determined in vehicle control and dosage groups at the different times following injection of collagenase relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are set at 100%.

Percent inhibition of thrombocytopenia is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

REFERENCES AND FURTHER READING

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B.4.16

Reversible Intravital Aggregation of Platelets

PURPOSE AND RATIONALE

Isotopic labeling of platelets can be employed to monitor platelet aggregation and desegregation *in vivo*. Adenosine diphosphate (ADP), platelet activating factor (PAF), arachidonic acid, thrombin and collagen are known to induce platelet aggregation. In the following procedure, labelled platelets are continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation promoting agents produces an increase in counts in A and a fall in counts in B. This observation implies that platelets are being aggregated within the vascular system and accumulate in the pulmonary microvasculature. The *in vivo* method can be used to evaluate platelet anti-aggregatory properties of test compounds.

PROCEDURE

Preparation of Labeled Platelets

Blood is obtained from rats by cardiopuncture. After centrifugation at 240 g for 10 min, the platelet-rich plasma (PRP) is transferred into a tube and suspended in calcium-free Tyrode solution containing 250 ng/ml PGE₁. The suspension is centrifuged at 640 g for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking with calcium-free Tyrode solution containing 250 ng/ml PGE₁. ⁵¹Cr is added to 1 ml of the platelet suspension. Following a 20-min incubation period at 37°C, the suspension is again centrifuged at 640 g for 10 min. The supernatant

is removed, and the labeled platelets are finally resuspended in 1 ml calcium-free Tyrode solution containing 250 ng/ml PGE₁.

In Vivo Experiment

Male Sprague-Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anaesthetized with pentobarbital sodium (30 mg/kg, i.p.). Following tracheotomy, the vena femoralis is exposed and cannulated. The labeled platelets are administered via the cannula. The circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. The counts are collected using a dual-channel gamma spectrometer (Nuclear Enterprise 4681) incorporating a microcomputer (AM 9080A). One hour after administration of labeled platelets (when counts in A and B have stabilized), the aggregation-promoting agent (ADP, PAF, arachidonic acid, thrombin or collagen) is administered twice by intravenous injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 hours after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (ADP, PAF, arachidonic acid, thrombin), or 1 hour (collagen) after compound administration another control injection of the aggregating agent is given. This injection is either used as an additional control or it may reveal long-term efficacy of a test compound.

EVALUATION

The microcomputer continuously reveals information about aggregation and desegregation of labeled platelets.

The following parameters are recorded:

A = counts over thorax

B = counts over abdomen

Difference: *A*–*B*

ratio: *A*/*B*.

The time course of response is shown in a curve. The area under the curve is calculated by a computer program.

Statistical significance is calculated using the Student's *t*-test.

MODIFICATION OF THE METHOD

Oyekan and Botting (1986) described a method for monitoring platelet aggregation *in vivo* in rats, using platelets labeled with indium³⁺ oxine and recording the increase in radioactivity count in the lung after injection of adenosine diphosphate or collagen.

Smith et al. (1989) monitored continuously the intrathoracic content of intravenously injected ¹¹¹indium-labeled platelets in anesthetized guinea pigs using a microcomputer-based system.

REFERENCES AND FURTHER READING

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B.5 Bleeding Models

B.5.1

Subaqueous Tail Bleeding Time in Rodents

PURPOSE AND RATIONALE

The damage of a blood vessel results in the formation of a hemostatic plug, which is achieved by several different mechanisms including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot.

A diagnostic parameter for specific defects of the hemostatic system and for the influence of drugs affecting hemostasis is the length of time that it takes for bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. The transection of the tail of a rodent was first established by Döttl and Ripke (1936) and is commonly used in experimental pharmacology.

PROCEDURE

Anaesthetized rats are fixed in supine position on a temperature-controlled (37°C) heating table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, the test compound is administered. After a defined latency period, the tail of the rat is transected with a razor-blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37°C).

EVALUATION

The time until bleeding stops is determined within a maximum observation time of 600 sec.

CRITICAL ASSESSMENT OF THE METHOD

There are numerous variables that can influence rodent's bleeding time measurements, as discussed by Dejana et al. (1979): position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, or procedure of injury (Simplate method, transsection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay condition (Stella et al. 1975; Minsker and Kling 1977).

Furthermore, it is impossible to transect exactly one blood vessel, because the transected tail region consists of a few major arteries and veins with mutual interaction between one another.

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B.5.2

Arterial Bleeding Time in Mesentery

PURPOSE AND RATIONALE

Arterial bleeding is induced by micropuncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in living blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and to fibrin formation. In this test, compounds are evaluated that inhibit thrombus formation, thus prolonging arterial bleeding time. The test is used to detect agents which interfere with primary hemostasis in small arteries.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), the animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats are placed on an electrically warmed table at 37°C.

The abdomen is opened by a mid-line incision and the mesentery is lifted to display the mesenteric arter-

ies. The mesentery is draped over a plastic plate and superfused continuously with Tyrode's solution maintained at 37°C. Bleeding times are determined with small mesenteric arteries (125–250 µm external diameter) at the junction of mesentery with intestines. Adipose tissue surrounding the vessels is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge: 16 × 5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40×. The time in seconds is determined from the puncturing until the bleeding is arrested by a hemostatic plug.

EVALUATION

1. Mean values of bleeding times are determined for each dosage group (4–6 animals, 4–6 punctures each) and compared to the controls.
 2. The significance of the results is assessed with the unpaired Student's *t*-test.
 3. The percent prolongation of bleeding time in dosage groups relative to the vehicle controls is calculated.
- For further details on methods and evaluations of various mechanisms or agents see the following: Butler et al. (1982), Dejana et al. (1979), and Zawilska et al. (1982).

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B.5.3

Template Bleeding Time Method

PURPOSE AND RATIONALE

The template bleeding time method is used to produce a standardized linear incision into the skin of humans to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system. The method has been modified with the development of a spring-loaded cassette with two disposable blades (Simplate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility of length and depth of dermal incisions. Forsythe and Willis (1989) described a method that enables the Simplate tech-

nique to be used as a method to analyze the bleeding time in the oral mucosa of dogs.

PROCEDURE

The dog is positioned in sternal or lateral recumbency. A strip of gauze is tied around both the mandible and maxilla as a muzzle. The template device is placed evenly against the buccal mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1, Fisher Scientific Co, Clifton, NJ) held directly below, but not touching the wounds. The position of the filter paper is changed every 15 sec. The end point for each bleeding is determined when the filter paper no longer develops a red crescent.

EVALUATION

The time from triggering the device until blood no longer appears on the paper is recorded as the bleeding time. The normal range lies between 2 to 4 min.

CRITICAL ASSESSMENT OF THE METHOD

The template bleeding time varies considerably between laboratories as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times in dogs have been recognized with thrombocytopenia, von Willebrand's disease, uremia, treatment with aspirin, anticoagulants, and dextran (Forsythe and Willis 1989, Klement et al. 1998). Brassard and Meyers (1991) describe the buccal mucosa bleeding time as a test that is sensitive to platelet adhesion and aggregation deficits. Generally, results of antithrombotic drugs in bleeding time models in animals do not exactly predict bleeding risks in clinical situations. But the models allow comparison between drugs with different actions (Dejana et al. 1979, Lind 1991).

MODIFICATIONS OF THE METHOD

The Simplate device can also be used to perform incisions at the shaved inner ear of rabbits, taking care to avoid major vessels. The normal range of bleeding time in anaesthetized rabbits is approximately 100 sec.

Klement et al. (1998) described another ear bleeding model in anaesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37°C. Five full-thickness cuts were made with a no. 11 Bard-Parker scalpel blade, avoiding major vessels, and the ear was immediately re-immersed in saline. At different times thereafter (5 to 30 min) aliquots of the saline

solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (1982). A guillotine-type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick, to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. The normal range lies between 2 to 8 min.

Kubitza et al. (2005) and others extended the use of the model in humans.

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B.6

Genetic Models of Hemostasis and Thrombosis

PURPOSE AND RATIONALE

Recent advances in genetic molecular biology have provided tools allowing scientists to design genetically altered animals that are deficient in certain proteins involved in thrombosis and hemostasis (so-called “knock-outs”, or “nulls”) (Carmeliet and Collen 1999, Pearson and Ginsburg 1999). These animals have been extremely useful for identifying and validating novel targets for therapeutic intervention. That is, by examin-

ing the phenotype (e.g. spontaneous bleeding, platelet defect, prolonged bleeding after surgical incision, etc.) of a specific knock-out strain, scientists can identify the role of the knocked-out protein. Then if the phenotype is favorable (e.g. not lethal), pharmacological agents can be designed to mimic the knock-out. More recently, novel gene medicine approaches have also benefited greatly from the availability of these models, as discussed below. The following section briefly summarizes some of the major findings in thrombosis and hemostasis using genetically altered mice and concludes with an example of how these models have been used in the drug discovery process.

The majority of these gene knock-outs result in mice that develop normally, are born in the expected Mendelian ratios, and are viable (as defined by the ability to survive to adulthood). Although seemingly normal, these knock-out mice display alterations in hemostatic regulation, especially when challenged. Deletion of FVIII, FIX, vWF, and the β_3 -integrin (Bi et al. 1996, Denis et al. 1998, Hodivala-Dilke et al. 1999, Wang et al. 1997) all result in mice that bleed upon surgical challenge, and despite some minor differences in bleeding susceptibility, these mouse knock-out models mirror the human disease states quite well (hemophilia A, hemophilia B, von Willebrand disease, and Glanzmann's thrombasthenia, respectively). In addition, deletion of some hemostatic factors results in fragile mice with severe deficiencies in their ability to regulate blood loss. Prenatally, these mice appear to develop normally, but they are unable to survive the perinatal period due to severe hemorrhage, in most cases due to the trauma of birth.

Genetic knock-outs have also been useful in dissecting the role of individual signaling proteins in platelet activation. Deletion of the β_3 -integrin (Hodivala-Dilke et al. 1999) or of G_{α_q} (Offermanns et al. 1997) results in dramatic impairment of agonist-induced platelet aggregation. Alteration of the protein-coding region in the β_3 -integrin carboxy-tail, β_3 -DiY, at sites that are thought to be phosphorylated upon platelet activation, also results in unstable platelet aggregation (Law et al. 1999). Deletion of various receptors such as thromboxane A_2 , P-selectin, P2Y1, and PAR-3 demonstrate diminished responses to some agonists while other platelet responses are intact (Thomas et al. 1998, Subramaniam et al. 1996, Leon et al. 1999, Kahn et al. 1998). Deletion of PAR-3, another thrombin receptor in mice, has little effect on hemostasis. This indicated the presence of yet another thrombin receptor in platelets and led to the identification of PAR-4 (Kahn et al. 1998).

Given that knock-outs of prothrombotic factors yield mice with bleeding tendencies, it follows that deletion of factors in the fibrinolytic pathway results in increased thrombotic susceptibility in mice. Plasminogen (Bugge et al. 1995; Ploplis et al. 1995), tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and the combined t-PA/u-PA knock-out (Carmeliet et al. 1994) result in mice that demonstrate impaired fibrinolysis, susceptibility for thrombosis, vascular occlusion, and tissue damage due to fibrin deposition. Interestingly, due to fibrin formation in the heart, these mice may provide a good model of myocardial infarction and heart failure caused by thrombosis (Christie et al. 1999). Intriguingly, mice deficient in PAI-1, the primary inhibitor of plasminogen activator, demonstrate no spontaneous bleeding and a greater resistance to venous thrombosis due to a mild fibrinolytic state (Carmeliet et al. 1993), suggesting that inhibition of PAI-1 might be a promising approach for novel antithrombotic agents.

In addition to their role in the regulation of hemostasis, several of these genes are important in embryonic development. For example, deletion of tissue factor (Bugge et al. 1996; Toomey et al. 1996; Carmeliet et al. 1996), tissue factor pathway inhibitor (Huang et al. 1997), or thrombomodulin (Healy et al. 1995) results in an embryonic lethal phenotype. These and other (Connolly et al. 1996, Cui et al. 1996) hemostatic factors also appear to contribute to vascular integrity in the developing embryo. These data suggest that initiation of coagulation and generation of thrombin is important at a critical stage of embryonic development, yet other factors must contribute since some of these embryos are able to progress and survive to birth.

Clearly, genetically altered mice have provided valuable insight into the roles of specific hemostatic factors in physiology and pathophysiology. Results of these studies have provided rationale and impetus for attacking certain targets pharmacologically. These types of models have also provided excellent model systems for studying novel treatments for human diseases. For example, these models provided exceptional systems for studying gene therapy for hemophilia. Specifically, deletion of FIX, generated by specific deletions in the *FIX* gene and its promoter, results in mice that mimic the human phenotype of hemophilia B (Lin et al. 1997). When these mice are treated with adenoviral mediated transfer of human FIX, the bleeding diathesis is fully corrected (Kung et al. 1998). Similarly, selectively bred dogs that have a characteristic point mutation in the sequence encoding the catalytic domain of FIX also have a severe hemophilia B that is

phenotypically similar to the human disease (Evans et al. 1989). When adeno-associated virus-mediated canine FIX gene was administered to these dogs intramuscularly, therapeutic levels of FIX were measured for up to 17 months (Herzog et al. 1999). Clinically relevant partial recovery of whole blood clotting time and APTT was also observed over this prolonged period. These data provided support for initiating the first study of adeno-associated virus-mediated FIX gene transfer in humans (Kay et al. 2000). Preliminary results from this clinical study provided evidence for expression of FIX in the three hemophilia patients studied and also provided favorable safety data to substantiate studying this therapy at higher doses. Although it is likely that there are differences between the human disease and animal models of hemophilia (or other diseases), it is clear that these experiments have provided pharmacological, pharmacokinetic, and safety data that were extremely useful in developing this approach and designing safe clinical trials.

Gene therapy approaches to rescuing patients with bleeding diatheses are further advanced than gene therapy for thrombotic indications. However, promising preclinical data indicates that local overexpression of thrombomodulin (Vaugh et al. 1999a) or tissue plasminogen activator (Vaugh et al. 1999b) inhibits thrombus formation in a rabbit model of arterial thrombosis. Similarly, local gene transfer of tissue factor pathway inhibitor prevented thrombus formation in balloon-injured porcine carotid arteries (Zoldhelyi et al. 2000). These and other studies (Vassalli et al. 1997) suggest that novel gene therapy approaches will also be effective for thrombotic indications, but these treatments will need to be carefully optimized for pharmacokinetics, safety and, efficacy in laboratory animal studies prior to administration to humans.

Genetically Modified Animals

Development and application of animal models of thrombosis has played a crucial role in discovering and validating novel drug targets, selecting new agents for clinical evaluation, and providing dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. The development and application of small and large animal models of thrombosis to the discovery and development of novel antithrombotic agents is described in this review. The methods and major issues regarding the use of animal models of thrombosis, such as positive controls, appro-

priate pharmacodynamic markers of activity, safety evaluation, species-specificity, and pharmacokinetics, are highlighted. Finally, the use of genetic models of thrombosis/hemostasis is presented using gene-therapy for hemophilia as an example of how animal models have aided in the development of therapies that are presently being evaluated clinically.

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B.6.1

Knock-Out Mice

PURPOSE AND RATIONALE

Genetically modified animals, in particular knock-out mice, help in the understanding of the role of various factors in blood clotting, thrombolysis, and platelet function. They are useful to verify the mode of action of new drugs.

Factor I (Fibrinogen)

Phenotype

Born in normal appearance, ~10% die shortly after birth and another 40% around 1–2 months after birth due to bleeding, failure of pregnancy, blood samples failing to clot or support platelet aggregation *in vitro* (Suh et al. 1995).

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Factor II (Prothrombin)

Phenotype

Partial embryonic lethality: 50% between embryonic day (E) 9.5–11.5; at least 1/4 survive to term, but fatal hemorrhage few days after birth; factor II important in maintaining vascular integrity during development as well as postnatal life (Sun et al. 1998, Xu et al. 1998).

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Factor V

Phenotype

Half of the embryos die at E9–10, possibly as a result of abnormal yolk sac vasculature, remaining 50% progress normally to term, but die from massive hemorrhage within 2 hours of birth, more severe in mouse than in human (Cui et al. 1996; Yang et al. 2000).

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Factor VII

Phenotype

Develop normally but suffer fatal perinatal bleeding (Rosen et al. 1997).

REFERENCES AND FURTHER READING

- Rosen ED, Chan JCY, Idusogie E et al (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature* 390:290–294

Factor VIII**Phenotype**

Mild phenotype compared with severe hemophilia A in humans; no spontaneous bleeding, illness or reduced activity during the first year of life; have residual clotting activity (APTT), as shown by Bi et al. (1995).

REFERENCES AND FURTHER READING

Bi L, Lawler AM, Antonarakis SE et al (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 10:119–121

Factor IX**Phenotype**

Factor IX coagulant activities (APTT): +/+ 92%, +/- 53%, -/- <5%; bleeding disorder (extensive bleeding after clipping a portion of the tail, bleeding to death if not cauterized (Kundu et al. 1998, Wang et al. 1997).

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Wang L, Zoppe M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. *Proc Natl Acad Sci USA* 94:11563–11566

Factor X**Phenotype**

Partial embryonic lethality (1/3 died on E11.5–12.5); fatal neonatal bleeding between postnatal day (P) 5–20, as shown by Dewerchin et al. (2000).

REFERENCES AND FURTHER READING

Dewerchin M, Liang Z, Moons L et al (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. *Thromb Haemost* 83:185–190

Factor XI**Phenotype**

APTT prolonged in -/- (158–200 sec) compared with +/+ (25–34 sec) and +/- (40–61 sec); no factor XI activity and antigen, did not result in intrauterine death, -/- similar bleeding as +/+ with a tendency to prolongation (Gailani et al. 1997).

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Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. *Blood Coagul Fibrinolysis* 8:134–144

TF (Tissue Factor)**Phenotype**

Abnormal circulation from yolk sac to embryo ~E8.5 leading to embryo wasting and death; TF has a role

in blood vessel development (Bugge et al. 1996, Carmeliet et al. 1996, Toomey et al. 1996, 1997).

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TFPI (Tissue Factor Pathway Inhibitor)**Phenotype**

None survive the neonatal period; 60% die between E9.5–11.5 with signs of yolk sac hemorrhage (Huang et al. 1997).

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Thrombin Receptor**Phenotype**

50% die at E9–10; 50% survive and become grossly normal adult mice with no bleeding diathesis; -/- platelets strongly respond to thrombin; -/- fibroblast lose their ability to respond to thrombin → second TR must exist, as shown by Connolly et al. (1996) and by Darrow et al. (1996).

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Darrow AL, Fung-Leung WP, Ye RD et al (1996) Biological consequences of thrombin receptor deficiency in mice. *Thromb Haemost* 76:860–866

Thrombomodulin**Phenotype**

Embryonic lethality before development of a functional cardiovascular system; die before E9.5 due to retardation of growth; TM+/- mice develop normal without thrombotic complications (Christie et al. 1999, Healy et al. 1995, 1998, Weiler-Guettler et al. 1998).

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Protein C**Phenotype**

KO mice appeared to develop normally macroscopically, but possessed obvious signs of bleeding and thrombosis; did not survive beyond 24 hours after delivery; microvascular thrombosis in the brain and necrosis in the liver; plasma clottable fibrinogen was not detectable, suggesting fibrinogen depletion and secondary consumptive coagulopathy (Jalbert et al. 1998).

REFERENCES AND FURTHER READING

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Plasminogen**Phenotype**

Severe spontaneous thrombosis; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; impaired skin healing; reduced macrophage and keratinocyte migration (Bugge et al. 1995, Ploplis et al. 1995).

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Alpha₂-Antiplasmin**Phenotype**

Normal fertility, viability and development; no bleeding disorder; spontaneous lysis of injected clots; → enhanced fibrinolytic potential; significant reduction of renal fibrin deposition after LPS (Lijnen et al. 1999).

REFERENCES AND FURTHER READING

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T-PA (Tissue-Type Plasminogen Activator)**Phenotype**

Extensive spontaneous fibrin deposition; severe spontaneous thrombosis; impaired neointima formation; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; abnormal tissue remodeling (Carmeliet et al. 1998, Christie et al. 1999).

REFERENCES AND FURTHER READING

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PAI-1 (Plasminogen Activator Inhibitor-1)**Phenotype**

Reduced thrombotic incidence; no bleeding; accelerated neo-intima formation; reduced lung inflammation; reduced atherosclerosis. Detailed studies on PAI-1 are reported by Carmeliet et al. (1993), Eitzman et al. (1996), Erickson et al. (1990), Kawasaki et al. (2000), and Pinsky et al. (1998).

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TAFI (Thrombin Activatable Fibrinolysis Inhibitor)

Not described.

Vitronectin**Phenotype**

Normal development, fertility, and survival; serum is completely deficient in “serum spreading factor” and PAI-1 binding activities; delayed arterial and venous thrombus formation (Eitzman et al. 2000, Zheng et al. 1995).

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Urokinase, U-PA (Urinary-Type Plasminogen Activator)**Phenotype**

Single u-PA deficiency: viable, fertile, normal life span; occasionally spontaneous fibrin deposits in normal and inflamed tissue; higher incidence of endotoxin-induced thrombosis. Combined t-PA and u-PA deficiency: mice survive embryonic development; retarded growth, reduced fertility, shortened life span; spontaneous fibrin deposits more extensively and in more organs (Carmeliet et al. 1998, Heckel et al. 1990).

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intraabdominal bleeding directly related to transgene expression in the liver and elevated plasma u-PA level; 50% die between 3 and 84 hours after birth; severe hypofibrinogenemia, loss of clotting function.

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UPAR (Urinary-Type Plasminogen Activator Receptor)**Phenotype**

Phenotype normal; attenuated thrombocytopenia and mortality associated with severe malaria (Bugge et al. 1995, 1996, Dewerchin et al. 1996, Piguet et al. 2000).

REFERENCES AND FURTHER READING

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Gas 6 (Growth Arrest-Specific Gene 6 Product)**Phenotype**

Mice are viable, fertile, appear normal; do not suffer spontaneous bleeding or thrombosis; have normal tail bleeding time. Platelets fail to aggregate irreversibly to ADP, collagen, or U 46619. Arterial and venous thrombosis is inhibited and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine (Angelillo-Scherrer et al. 2001).

REFERENCES AND FURTHER READING

- Angelillo-Scherrer A, DeFrutos PG, Aparicio C et al (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med* 7:215–221

GPIbalpha (Glycoprotein Ib Alpha, Part of the GP Ib-V-IX Complex)**Phenotype**

Bleeding, thrombocytopenia and giant platelets (similar to human Bernard Soulier syndrome). See Ware et al. (2000) for details.

REFERENCES AND FURTHER READING

- Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. *Proc Natl Acad Sci USA* 97:2803–2808

GPV (Glycoprotein V, Part of the GP Ib-V-IX Complex)**Phenotype**

Increased thrombin responsiveness, GpV^{-/-} platelets are normal in size, normal amounts in GpIb-IX, functional in vWF-binding; platelets are hyperresponsive to thrombin → increased aggregation response; shorter bleeding time; → GpV = negative modulator of platelet function (Ramakrishnan et al. 1999).

REFERENCES AND FURTHER READING

- Ramakrishnan V, Reeves PS, DeGuzman F et al (1999) Increased thrombin responsiveness in platelets from mice

lacking glycoprotein V. Proc Natl Acad Sci USA 96:13336–13341

GP1Ib (Integrin Alpha 1Ib, Glycoprotein 1Ib, Part of the GP 1Ib–1Ila Complex)

Phenotype

Bleeding disorder similar to Glanzmann thrombasthenia in man; platelets failed to bind fibrinogen, to aggregate and to retract a fibrinogen clot; α -granules do not contain fibrinogen (Tronik-Le Roux et al. 2000).

REFERENCES AND FURTHER READING

Tronik-Le Roux D, Roullot V, Poujol C et al (2000) Thrombasthenic mice generated by replacement of the integrin α_{1Ib} gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

GP 1Ila (Integrin Beta3, Glycoprotein 1Ila, Part of the GP 1Ib–1Ila Complex)

Phenotype

Viable, fertile, increased fetal mortality; features of Glanzmann thrombasthenia in man, e.g. defective platelet aggregation, clot retraction; spontaneous bleeding, prolonged bleeding times; dysfunctional osteoclasts, development of osteosclerosis with age (Hodivala-Dilke et al. 1999, McHugh et al. 2000).

REFERENCES AND FURTHER READING

Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238
 McHugh KP, Hodivala-Dilke K, Zheng MH et al (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

GP 1Ia (Glycoprotein 1Ia, Integrin Beta 1, Part of the GP 1a–1Ia Complex)

Phenotype

Integrin beta1 null platelets from conditional knockout mice develop normally, platelet count is normal. Collagen induced platelet aggregation is delayed but otherwise normal; tyrosine phosphorylation pattern is normal but phosphorylation is delayed. Bleeding time in bone marrow chimeric mice is normal; no major *in vivo* defects (Nieswandt et al. 2001).

REFERENCES AND FURTHER READING

Nieswandt B, Brakebusch C, Bergmeier W et al (2001) Glycoprotein VI but not $\alpha_2\beta_1$ integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

VWF (von Willebrand Factor)

Phenotype

Factor VIII levels strongly reduced due to defective protection by vWF; highly prolonged bleeding time, hemorrhage, spontaneous bleeding; mice useful for investigating the role of vWF; delayed platelet adhesion in ferric-chloride-induced arteriolar injury (Denis et al. 1998, Ni et al. 2000).

REFERENCES AND FURTHER READING

Denis C, Methia N, Frenette PS et al (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:952–959
 Ni H, Denis CV, Subbarao S et al (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 106:385–392

Thromboxane A2 Receptor (TXA2r)

Phenotype

Mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid (Thomas et al. 1998).

REFERENCES AND FURTHER READING

Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 102:1994–2001

Prostacyclin Receptor (PGI2r)

Phenotype

Viable, fertile, normotensive; increased susceptibility to thrombosis; reduced inflammatory and pain responses (Murata et al. 1997).

REFERENCES AND FURTHER READING

Murata T, Ushikubi F, Matsuoka T et al (1997) Altered pain reception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

PECAM (Platelet: Endothelial Cell Adhesion Molecule)

Phenotype

Normal platelet aggregation; prolonged bleeding time as described by Duncan et al. (1999) and by Mahooti et al. (2000).

REFERENCES AND FURTHER READING

Duncan GS, Andrew DP, Takimoto H et al (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1) CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162:3022–3030
 Mahooti S, Graesser D, Patil S et al (2000) PECAM-1 (CD 31) expression modulates bleeding time *in vivo*. Am J Pathol 157:75–81

Pallid (Pa)**Phenotype**

Among 13 hypopigment mouse mutants with storage pool deficiency, the pallid mouse is a model of the human Hermansky Pudlak syndrome (the beige mouse is a model of the Chediak Higashi syndrome). Pallid mice exhibit prolonged bleeding time, pigment dilution, kidney lysosomal enzyme elevation serum alpha 1 antitrypsin deficiency and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino acid protein pallidin, which interacts with syntaxin 13, a protein mediating vesicle docking and fusion (Huang et al. 1999).

REFERENCES AND FURTHER READING

Huang L, Kuo YM, Gitschier J (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. *Nat Genet* 23:329–332

G Alpha(q) (Guanyl Nucleotide Binding Protein G Alpha q) Phenotype

Defective aggregation in response ADP, TXA₂, thrombin, collagen; shape change normal (Offermans et al. 1997, Ohlmann et al. 2000).

REFERENCES AND FURTHER READING

Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. *Nature* 389:183–186

Ohlmann P, Eckly A, Freund M et al (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood* 96:2134–2139

G z (Member of the Gi Family of G Proteins)**Phenotype**

Impaired platelet aggregation to epinephrine; resistance to fatal thromboembolism; exaggerated response to cocaine, reduced effect of morphine and antidepressant drugs (Yang et al. 2000).

REFERENCES AND FURTHER READING

Yang J, Wu J, Kowalska MA et al (2000) Loss of signaling through G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci USA* 97:9984–9989

Phospholipase C Gamma**Phenotype**

Viable, fertile, decreased mature B cells; defective B cell and mast cell function; defective Fc_{gamma} receptor

signaling, therefore, loss of collagen-induced platelet aggregation (Wang et al. 2000).

REFERENCES AND FURTHER READING

Wang D, Feng J, Wen R et al (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity* 13:25–35

CD39 (Vascular Adenosine Triphosphate Diphosphohydrolase)**Phenotype**

Viable, fertile; prolonged bleeding times but minimally perturbed coagulation parameters; reduced platelet interaction with injured mesenteric vasculature *in vivo*. Platelets fail to aggregate to standard agonists *in vitro* associated with purinergic P2Y₁ receptor desensitization; fibrin deposition at multiple organ sites (Enjyoji et al. 1999).

REFERENCES AND FURTHER READING

Enjyoji K, Seigny J, Lin Y et al (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med* 5:1010–1017

Protein Kinase, cGMP-Dependent**Phenotype**

Viable, fertile; unresponsive to cGMP and NO; defective VASP-phosphorylation; increased adhesion and aggregation of platelets *in vivo* in ischemic/reperfused mesenteric microcirculation; no compensation by cAMP kinase system (Massberg et al. 1999).

REFERENCES AND FURTHER READING

Massberg S, Sausbier M, Klatt P et al (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. *J Exp Med* 189:1255–1264

Vasodilator-Stimulated Phosphoprotein (VASP)**Phenotype**

Viable, fertile; mild platelet dysfunction with megakaryocyte hyperplasia, increased collagen/thrombin activation, impaired cyclic nucleotide mediated inhibition of platelet activation (Aszodi et al. 1999, Hauser et al. 1999).

REFERENCES AND FURTHER READING

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Hauser W, Knobloch KP, Eigenthaler M et al (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knock-out mice. *Proc Natl Acad Sci USA* 96:8120–8125

Arachidonate 12-Lipoxygenase (P-12LO)**Phenotype**

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in *ex vivo* assays and increased mortality in an ADP-induced mouse model of thromboembolism (Chen et al. 1994, Johnson et al. 1998).

REFERENCES AND FURTHER READING

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Arachidonate 5-Lipoxygenase (P-5LO)**Phenotype**

Develop normally and are healthy. No difference in their reaction to endotoxin shock; however, they resist the lethal effects of shock induced by platelet-activating factor. Inflammation induced by arachidonic acid is markedly reduced (Chen et al. 1994, Johnson et al. 1998).

REFERENCES AND FURTHER READING

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- Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. *Proc Natl Acad Sci USA* 95:3100–3105

Thrombopoietin**Phenotype**

TPO $-/-$ and *c-mpl* $-/-$: both exhibit a 90% reduction in megakaryocyte and platelet levels, but even with these small platelet levels the mice do not have excessive bleeding; all platelets that are present are morphologically normal+functionally; *in vivo* TPO is required for control of megakaryocyte and platelet number but not for their maturation (Lawler et al. 1998).

REFERENCES AND FURTHER READING

- Lawler J, Sunday M, Thibert V et al (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest* 101:982–992

Thrombospondin-1**Phenotype**

Normal thrombin-induced platelet aggregation; increase in circulating number of white blood cells; TSP-

1 is involved in normal lung homeostasis (Lawler et al. 1998).

REFERENCES AND FURTHER READING

- Lawler J, Sunday M, Thibert V, et al (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest* 101:982–992

Mouse knock-out models of virtually all of the known hemostatic factors have been reported, as shown in Table 6.

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- Jalbert LR, Rosen ED, Moons L et al (1998) Inactivation of the gene for anticoagulant protein C causes lethal peri-

Table 6 Genetic models of thrombosis and hemostasis

| Knock-out | Viable | Embryonic Development / Survival | References |
|---------------------------|--------|-----------------------------------------|-----------------------------------------|
| Coagulation | | | |
| Protein C | No | Normal Perinatal death | Jalbert et al. 1998 |
| Fibrinogen | Yes | Normal Perinatal death | Suh et al. 1995 |
| Fibrinogen-QAGVD | Yes | Normal | Suh et al. 1995 |
| fV | No | Partial embryonic loss Perinatal death | Cui et al. 1996 |
| FVII | Yes | Normal Perinatal death | Rosen et al. 1997 |
| fVIII | Yes | Normal | Bi et al. 1996 |
| fIX | Yes | Normal | Wang et al. 1997 |
| fXI | Yes | Normal | Gailani et al. 1997 |
| Tissue factor | No | Lethal | Toomey et al. 1996 Bugge et al. 1996 |
| TFPI | No | Lethal | Huang et al. 1997 |
| vWF | Yes | Normal | Denis et al. 1998 |
| Prothrombin | No | Partial embryonic lossr perinatal death | Xue et al. 1998 Sun et al. 1998 |
| Fibrinolytic | | | |
| u-Pa & t-PA | Yes | Normal Growth retardation | Carmeliet et al. 1994 |
| uPAR | Yes | Normal | Dewerchin et al. 1996 Bugge et al. 1995 |
| Plasminogen | Yes | Normal Growth retardation | Bugge et al. 1995 Ploplis et al. 1995 |
| PA-I | Yes | Normal | Carmeliet et al. 1993 |
| Thrombomodulin | No | Lethal | Healy et al. 1995 |
| Platelet | | | |
| β_3 | Yes | Normal Partial embryonic loss | Hodivala-Dilke et al. 1999 |
| β_3 -DiYF | Yes | Normal | Law et al. 1999 |
| P-Selectin | Yes | Normal | Subramaniam et al. 1996 |
| PAR-1 | Yes | Normal | Connolly et al. 1996 |
| PAR-3 | Yes | Normal | Kahn et al. 1998 |
| $G_{\alpha a}$ | Yes | Normal Perinatal death | Offermans et al. 1997 |
| TXA ₂ receptor | Yes | Normal | Thomas et al. 1998 |
| P2Y1 | Yes | Normal | Leon et al. 1999 |

- natal consumptive coagulopathy in mice. *J Clin Invest* 102:1481–1488
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- Leon C, Hechler B, Freund M et al (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y₁ receptor-null mice. *J Clin Invest* 104:1731–1737
- Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G α (q)-deficient mice. *Nature* 389:183–186
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- Subramaniam M, Frenette PS, Saffaripour S et al (1996) Defects in hemostasis in P-selectin-deficient mice. *Blood* 87:1238–1242
- Suh TT, Holmback K, Jensen NJ et al (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes Dev* 9:2020–2033
- Sun WY, Witte DP, Degen JL et al (1998) Prothrombin deficiency results in embryonic and neonatal lethality in mice. *Proc Natl Acad Sci USA* 95:7597–7602
- Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A₂. *J Clin Invest* 102:1994–2001
- Toomey JR, Kratzer KE, Lasky NM et al (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. *Blood* 88:1583–1587
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B.7 Critical Issues in Experimental Models

B.7.1 The Use of Positive Control

Clearly, there are many antithrombotic agents that can be used to compare and contrast the antithrombotic efficacy and safety of novel agents. The classic antithrombotic agents are heparin, warfarin, and aspirin. However, new, more selective agents such as hirudin, low molecular weight heparins, and clopidogrel are commercially available that will either replace or augment these older treatments. Novel antithrombotic agents should certainly be demanded to demonstrate better efficacy than currently available therapy in animal models of thrombosis. This should be demonstrated by performing dose-response experiments that include maximally effective doses of each compound in the model. At the maximally effective dose, parameters such as APTT, PT, template bleeding time, or other, more sensitive measurements of systemic hypocoagulability or bleeding should be compared. A good example of this approach is a study by Schumacher et al. (1996), who compared the antithrombotic efficacy of argatroban and dalteparin in arterial and venous models of thrombosis. Consideration of potency and safety compared to other agents should be taken into account when advancing a drug through the testing funnel.

The early *in vivo* evaluation of compounds that demonstrate acceptable *in vitro* potency and selectivity requires evaluation of each compound alone in order to demonstrate antithrombotic efficacy. The antithrombotic landscape is becoming complicated by so many agents from which to choose that it will become increasingly difficult to design preclinical experiments that mimic the clinical setting in which polyantithrombotic therapy is required for optimal efficacy and safety. Consequently, secondary and tertiary preclinical experiments will need to be carefully designed in order to answer these specific, important questions.

REFERENCES AND FURTHER READING

Schumacher WA, Heran CL, Steinbacher TE (1996) Low-molecular-weight heparin (Fragmin) and thrombin active-site inhibitor (argatroban) compared in experimental arterial and venous thrombosis and bleeding time. *J Card Pharmacol* 28:19–25

B.7.2 Evaluation of Bleeding Tendency

Although the clinical relevance of animal models of thrombosis has been well-established in terms of ef-

ficacy, the preclinical tests for evaluating safety, i.e., bleeding tendency, have not been as predictable. The difficulty in predicting major bleeding, such as intracranial hemorrhage, resulting from antithrombotic or thrombolytic therapy stems from the complexity and lack of understanding of the mechanisms involved in this disorder. Predictors of anticoagulant-related intracranial hemorrhages are advanced age, hypertension, intensity and duration of treatment, head trauma, and prior neurologic disease (Stieg and Kase 1998, Sloan and Gore 1992). These risk factors are clearly difficult, if not impossible, to simulate in laboratory animals. Consequently, more general tests of anticoagulation and primary hemostasis have been employed.

Coagulation assays provide an index of the systemic hypocoagulability of the blood after administration of antithrombotic agents; however, as indicated earlier, the sensitivity and specificity of these assays varies from compound to compound, so these assays do not provide a consistent safety measure across all mechanisms of inhibition. Consequently, many laboratories have attempted to develop procedures that provide an indication of bleeding risk by evaluating primary hemostasis after generating controlled incisions in anesthetized animals. Some of the tests used in evaluating FXa inhibitors include template bleeding time, tail transection bleeding time, cuticle bleeding time, and evaluation of clinical parameters such as hemoglobin and hematocrit. Unfortunately, template bleeding tests, even when performed in humans, have not been good predictors of major bleeding events in clinical trials (Bernardi et al. 1993, Bick 1995, Rodgers and Levin 1990). However, these tests have been able to demonstrate relative advantages of certain mechanisms and agents over others. For example, hirudin, a direct thrombin inhibitor, appears to have a narrow therapeutic window when used as an adjunct to thrombolysis in clinical trials, producing unacceptable major bleeding when administered at 0.6 mg/kg, i.v. bolus, plus 0.2 mg/kg/hr (Antman et al. 1996, GUSTO Investigators 1996). When the dose of hirudin was adjusted to avoid major bleeding (0.1 mg/kg and 0.1 mg/kg/hr), no significant therapeutic advantage over heparin was observed. If the relative improvement in the ratio between efficacy and bleeding observed preclinically with Xa inhibitors compared to thrombin inhibitors such as hirudin is supported in future clinical trials, this will establish an important safety advantage for FXa inhibitors and provide valuable information for evaluating the safety of new antithrombotic agents in preclinical experiments.

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B.7.3**Selection of Models Based on Species-Dependent Pharmacology/Physiology**

As alluded to earlier, species selection for animal models of disease is often limited by the unique physiology of a particular disease target in different species or by the species specificity of the pharmacological agent for the target. For example, it was discovered relatively early in the development of platelet GPIIb/IIIa antagonists that these compounds were of limited use in rats (Cox et al. 1992) and that there was a dramatic species-dependent variation in the response of platelets to GPIIb/IIIa antagonists (Bostwick et al. 1996, Cook et al. 1993, Panzer-Knodle et al. 1993). This discovery led to the widespread use of larger animals (particularly in dogs, whose platelet response to GPIIb/IIIa antagonists resembles humans) in the evaluation of GPIIb/IIIa antagonists. Of course the larger animals required more compound for evaluation, which created a resource problem for medicinal chemists. This was especially problematic for companies that generated compounds by combinatorial parallel synthetic chemistry in which many compounds can be made, but usually in very small quantities. However, some pharmacologists devised clever experiments that partially overcame this problem. Cook et al. (1996) administered a GPIIb/IIIa antagonist orally and intravenously to rats, and then mixed the platelet-rich plasma from the treated rats with platelet-rich plasma from untreated dogs. The mixture was then evaluated in an agonist-induced platelet aggregation assay and the resulting inhibition of canine platelet aggrega-

tion (rat platelets were relatively unresponsive to this GPIIb/IIIa antagonist) was due to the drug present in the plasma obtained from the rat. Using this method, only a small amount of drug is required to determine the relative bioavailability in rats. However, the animal models chosen for efficacy in that report (guinea pigs and dogs) were selected based on their favorable platelet response to the GPIIb/IIIa antagonist.

Similarly for inhibitors of FXa, there are significant variations in the activity of certain compounds against FXa purified from plasma of different species and in plasma-based clotting assays using plasma from different species. DX-9065 is much more potent against human FXa ($K_i = 78$ nM) than against rabbit ($K_i = 102$ nM) and rat ($K_i = 1980$ nM) FXa. Likewise, in the PT assay, DX-9065a was very potent in human plasma (concentration required to double PT, $PT \times 2$, was $0.52 \mu\text{M}$) and in squirrel monkey plasma ($PT \times 2 = 0.46 \mu\text{M}$), but was much less potent in rabbit, dog, and rat plasma ($PT \times 2 = 1.5, 6.5, \text{ and } 22.2 \mu\text{M}$, respectively). Other FXa inhibitors have also demonstrated these species-dependent differences in activity (Tidwell et al. 1980, Nutt et al. 1991, Taniuchi et al. 1998). Regardless, the investigator must be aware of these differences so that appropriate human doses can be extrapolated from the laboratory animal studies.

Although in many cases the exact mechanism for the species-dependent differences in response to certain therapeutic agents remains unclear, these differences must be examined to determine the appropriate species to be used for preclinical pharmacological evaluation of each agent. This evaluation can routinely be performed by *in vitro* coagulation or platelet aggregation tests prior to evaluation in animal models.

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B.7.4

Selection of Models Based on Pharmacokinetics

Much debate surrounds the issue as to which species most resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single animal can precisely mimic the gastrointestinal characteristics of humans (Kararli 1995). Due to resource issues (mainly compound availability) and animal care and use considerations, small rodents, such as rats, are usually considered for primary *in vivo* evaluation of pharmacokinetics for novel agents. However, there is great reservation about moving a compound into clinical trials based on oral bioavailability data derived from rat experiments alone. Usually, larger animals such as dogs or non-human primates, which have similar gastrointestinal morphology compared to humans, are the next step in the evaluation of pharmacokinetics of new agents. The pharmacokinetic characteristics of FXa inhibitor, YM-60828, have been studied extensively in a variety of laboratory animals. YM-60828 demonstrated species-dependent pharmacokinetics, with oral bioavailability estimates of approximately 4%, 33%, 7%, and 20% in rats, guinea pigs, beagle dogs, and squirrel monkeys, respectively. Although these results suggest that YM-60828 has somewhat limited bioavailability, evaluating the pharmacokinetic profile of novel agents in a number of species (Sanderson et al. 1998) is a well-established approach used to aid in identifying compounds for advancement to human testing. That is, acceptable bioavailability in a number of species suggests that a compound will be bioavailable in humans. Which of the laboratory species adequately represents the bioavailability of a specific compound in humans can only be determined after appropriate pharmacokinetic evaluation in humans. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate

animal model for testing the antithrombotic efficacy of compounds because the ultimate proof-of-concept experiment is to demonstrate efficacy by the intended route of administration.

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B.7.5

Clinical Relevance of Data Derived from Experimental Models

Animal models of thrombosis have played a crucial role in the discovery and development of a number of compounds that are now successfully being used for the treatment and prevention of thrombotic diseases. The influential preclinical results using novel antithrombotics in a variety of laboratory animal experiments are listed in Table 7 below, along with the early clinical trials and results for each compound. This table intentionally omits many compounds that were tested in animal models of thrombosis, but failed to be successful in clinical trials or, for other reasons, did not become approved drugs. However, these negative outcomes would not have been predicted by animal models of thrombosis because the failures were generally due to other shortcomings of the drugs (e.g., toxicity, narrow therapeutic window, or undesirable pharmacokinetics or pharmacodynamics) that are not always clearly presented in the scientific literature due to proprietary restrictions in this highly competitive field.

Nonetheless, it is clear that animal models have supplied valuable information for investigators responsible for evaluating these drugs in humans, providing pharmacodynamic, pharmacokinetic, and safety data that can be used to design safe and efficient clinical trials. For detailed applications see the following references: Bugge et al. (1995), Bugge et al. (1996), Carmeliet et al. (1993), Carmeliet et al. (1994), Carmeliet et al. (1996), Christie et al. (1999), Connolly et al. (1996), Cui et al. (1996), Evans et al. (1989), Healy et al. (1995), Herzog et al. (1999), Hodivala-Dilke et al. (1999), Huang et al. (1997), Kahn et al. (1998), Kay et al. (2000), Kung et al. (1998), Law et al. (1999), Leon et al. (1999), Lin et al. (1997), Offermanns et al. (1997), Ploplis et al. (1995), Subramaniam et al. (1996), Thomas et al. (1998), Toomey et al.

Table 7 Animal models of thrombosis and their clinical correlates

| Compound | Preclinical Animal Model | Preclinical Results | Ref | Clinical Indication | Clinical Result | Ref |
|-----------------------------------------------------|-----------------------------------------------------------------------|------------------------------------------------------------|----------------------------------------|---------------------------------------------------|-------------------------------------------------------------------------------------------------|----------------------------|
| Recombinant tissue plasminogen activator (Activase) | Rabbit pulmonary artery thrombosis | Lysis of preformed pulmonary thrombus | Matsuo et al. 1981 | Acute myocardial infarction—thrombolysis | Improved recanalization | Collen et al. (1984) |
| Abciximab (ReoPro) | Canine coronary cyclic flow reduction | Significant inhibition of platelet-dependent thrombosis | Coller et al. 1986; Coller et al. 1989 | High-risk coronary angioplasty | Reduction in death, myocardial infarction, refractory ischemia, or unplanned revascularization | EPIC Investigators (1994) |
| Tirofiban (Aggrestat) | Canine coronary cyclic flow reduction | Significant inhibition of platelet-dependent thrombosis | Lynch et al. 1995; | Unstable angina | Reduction in death, myocardial infarction, refractory ischemia | PRISM Investigators (1998) |
| Eptifibatid (Integrilin) | TPA-induced coronary thrombolysis | Significant improvement in lysis of occlusive thrombus | Nicolini et al. 1994 | Acute myocardial infarction—thrombolysis with tPA | Improvement in incidence and speed of reperfusion | Ohman et al. 1997 |
| Enoxaparin (Lovenox) | Canine coronary cyclic flow reduction | Significant inhibition of platelet-dependent thrombosis | Lynch et al. 1995 | Unstable angina | Significant decrease in death, myocardial infarction, and need for revascularization at 30 days | Cohen et al. 1998 |
| Hirudin (Refludan) | Rabbit jugular vein thrombus growth | Inhibition of thrombus growth compared to standard heparin | Agnelli et al., 1990 | Deep vein thrombosis after total hip replacement | Significantly decreased rate of DVT | Eriksson et al. 1997 |
| Argatroban | Canine coronary artery electrolytic-injury (TPA-induced thrombolysis) | Accelerated reperfusion and prevented reocclusion | Fitzgerald et al. 1989 | Unstable angina | No episodes of MI during drug infusion | Gold et al. 1993 |

(1996), Vassalli et al. (1997), Waugh et al. (1999), and Zoldhelyi et al. (2000).

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B.8 Safety Assays in Thrombosis and Haemostasis

See Mousa (2006).

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Chapter C

Activity on Urinary Tract¹

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¹Contribution for this edition by S.A. Hart, review of second edition by M. Hropot, contributions to the former editions by R. Greger, H. Gögelein, M. Hropot and M. Bleich.

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C.1 Diuretic and Saluretic Activity

C.1.1 In Vitro Methods

C.1.1.1 Carbonic Anhydrase Inhibition In Vitro

PURPOSE AND RATIONALE

Acetazolamide (Diamox) was one of the first synthetic non-mercurial diuretics. The mode of action was found to be inhibition of carbonic anhydrase. Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible hydration (or hydroxylation) of CO_2 to form H_2CO_3 which dissociates non-enzymatically into HCO_3^- and H^+ . The enzyme is located within the cytoplasm and at the apical and basolateral membranes of proximal tubules as well as on the apical (luminal) surface of distal tubules and in the thick ascending limb of the loop of Henle. Its primary function is to enhance H^+ secretion into the urine. At least three isoenzymes, designated as I, II and III or A, B and C, are known to exist.

Carbonic anhydrase inhibitors were originally used as diuretics but are no longer used for this purpose except in limited circumstances (metabolic alkalosis accompanied by edema). Chronic inhibition of carbonic anhydrase results in significant hypokalemia and metabolic acidosis due to bicarbonate wasting and thus inhibition of carbonic anhydrase may be mechanistically important if these effects are noted in *in vivo* studies or clinically (Okusa and Ellison, 2000). Numerous isoenzymes exist, of which CA II is the most abundant in proximal tubules and erythrocytes. The activity of carbonic anhydrase in erythrocytes is significantly higher than that in the kidney, and the enzyme is easily harvested by lysing mammalian whole blood.

Thus, examination of the degree of inhibition of erythrocyte carbonic anhydrase may serve as a suitable surrogate for examination of effects on renal activity (Maren 1967; Armstrong et al. 1966).

In spite of the fact that newer diuretics are based on other modes of action, the test for inhibition of carbonic anhydrase should be performed for evaluation of a new compound. Moreover, the specific use of carbonic anhydrase inhibitors as antiglaucoma drugs has been described (Friedland and Maren 1984; Caprioli 1985). The mechanism by which carbonic anhydrase inhibitors lower intraocular pressure is through a reduction in aqueous humor formation, by affecting electrolyte and water balance in the nonpigmented ciliary epithelium (Friedland and Maren 1984; Caprioli 1985). Although many methods to measure carbonic anhydrase activity have been developed (Philpot and Philpot 1936), the micro method described by Maren (1960) is relatively simple, sensitive and reliable. The enzyme source are red cells, a rich source of the same isoenzymes found in the eye (Maren 1967; Armstrong et al. 1966; Wistrand et al. 1986; Wistrand and Knunutila 1980).

PROCEDURE

The analytical method is based on the catalysis of the conversion of CO_2 to H_2CO_3 by the enzyme, with resulting decrease in pH being monitored colorimetrically (Philpot and Philpot, 1936).

Materials and Solutions

- phenol red indicator solution:
12.5 mg phenol red/liter 2.6 mM NaHCO_3 ,
pH 8.3 + 218 mM Na_2CO_3
- 1 M sodium carbonate/bicarbonate buffer, pH 9.8
- Enzyme: Carbonic anhydrase from dog blood; Blood is collected into a heparinized tube and diluted 1:100 with deionized water.
- Equipment
 - Reaction vessel – custom made by Labglass Inc., Vineland, NJ, USA
 - Monostat bench mounted flowmeter
 - 30% CO_2 – M& G Gases, Branchburg, NJ, USA

Assay

CO_2 flow rate is adjusted to 30 (45) ml/min. The following solutions are added to the reaction vessel:

400 μl phenol red indicator solution

100 μl enzyme

200 µl H₂O or appropriate drug concentration after 3 min for equilibration:

100 µl carbonate/bicarbonate buffer is added.

The following parameters are determined in duplicate samples:

T_u = (uncatalyzed time) = time for the color change to occur in the absence of enzyme.

T_e = (catalyzed time) = time for the color change to occur in the presence of the enzyme.

$T_u - T_e$ = enzyme rate

T_i = enzyme rate in the presence of various concentrations of inhibitor

EVALUATION

Percent inhibition of carbonic anhydrase is calculated according to the following formula:

$$\% \text{Inhibition} = 1 - \frac{(T_u - T_e) - (T_i - T_e)}{T_u - T_e} \times 100$$

Standard data:

- Compound IC_{50} [M]
- Acetazolamide 9.0×10^{-9}
- Chlorothiazide 9.0×10^{-7}

CRITICAL ASSESSMENT OF THE METHOD

Determination of carbonic anhydrase inhibition is of value to characterize the activity spectrum of sulfonamide diuretics.

MODIFICATIONS OF THE METHOD

Landolfi et al. (1997) reported a modified procedure for the measurement of carbonic anhydrase activity. The measure of carbonic anhydrase activity is based on the rate of CO₂ hydration by the enzyme. Such transformation was monitored by a procedure which consists in the measure of time necessary for the pH of an appropriate buffer to decrease from 8 to 7.5 in the presence of a constant CO₂ flow: such time period is dose-dependently reduced by the addition of the enzyme and further modified in the presence of carbonic anhydrase inhibitory compounds. The time required for a specific change in pH can be determined electrochemically as opposed to colorimetrically. This requires the use of a well-calibrated pH meter with a fast-reacting electrode. Activity is expressed in en-

zyme units, defined as a doubling of the time needed for the pH change in the absence of enzyme. These methods are much more sensitive than the colorimetric determination (Maren, 1960; Landolfi et al., 1997; Lukasi, 2005).

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C.1.2

In Vivo Methods

C.1.2.1

Diuretic Activity in Rats (LIPSCHITZ Test)

PURPOSE AND RATIONALE

A method for testing diuretic activity in rats has been described by Lipschitz et al. (1943). The test is based on water and sodium excretion in test animals and compared to rats treated with a high dose of urea. The "Lipschitz-value" is the quotient between excretion by test animals and excretion by the urea control.

PROCEDURE

Male Wistar rats weighing 100–200 g are used. Three animals per group are placed in metabolic cages provided with a wire mesh bottom and a funnel to collect the urine. Stainless-steel sieves are placed in the funnel to retain feces and to allow the urine to pass. The rats are fed with standard diet (Altromin pellets) and water ad libitum. Fifteen hours prior to the experiment food and water are withdrawn. Three animals are placed in one metabolic cage. For screening procedures two groups of three animals are used for one dose of the test compound. The test compound is applied orally at a dose of 50 mg/kg in 5.0 ml water/kg body weight. Two groups of 3 animals receive orally 1 g/kg urea. Additionally, 5 ml of 0.9% NaCl solution per 100 g body weight are given by gavage. Urine excretion is recorded after 5 and after 24 h. The sodium content of the urine is determined by flame photometry. Active compounds are tested again with lower doses.

EVALUATION

Urine volume excreted per 100 g body weight is calculated for each group. Results are expressed as the "Lipschitz-value", i. e., the ratio T/U , in which T is the response of the test compound, and U , that of urea treatment. Indices of 1.0 and more are regarded as a positive effect. With potent diuretics, Lipschitz values of 2.0 and more can be found. Calculating this index for the 24 h excretion period as well as for 5 h indicates the duration of the diuretic effect. Similar to urine volume, quotients can be calculated for sodium excretion. Dose-response curves can be established using various doses. Loop diuretics are characterized by a steep dose-response curve. Saluretic drugs, like hydrochlorothiazide, show Lipschitz values around 1.8, whereas loop diuretics (or high ceiling diuretics) like furosemide, bumetanide or piretanide reach values of 4.0 and more.

CRITICAL ASSESSMENT OF THE METHOD

The Lipschitz test has been proven to be a standard method and a very useful tool for screening of potential diuretics.

MODIFICATIONS OF THE METHOD

The method has been modified in various ways by several authors. Cummings et al. (1960) recommended a sequential procedure with criteria for acceptance or rejection of test drugs. Kau et al. (1984) recommended a method for screening diuretic agents in the rat using normal saline (4% body weight) as hydrating fluid.

Homozygous Brattleboro rats exhibit symptoms of diabetes insipidus (Valtin et al. 1965). The condition is due to the failure of hypothalamic neurons to produce vasopressin, which is due to a single base point deletion in the vasopressin gene (Schmale and Richter 1984). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus is reversed by a vasopressin agonist (Laycock et al. 1994).

These animals can be used to study vasopressin agonism and antagonism and the aquaretic effects of synthetic drugs.

Klatt et al. (1975) described a method of collecting urine excreted by cats. On the basis of urine funnel used in rats, an appropriate larger metabolism cage made out of transparent, rigid polyvinyl chloride was used. The cage was improved by a built-in sieve cone which assured good separation of urine and feces. A device to measure and record the time and amount of voided urine was attached. Urine was collected in a vessel with a hose connection from the bottom to a pressure sensor. An attached overflow tube could be occluded. The initial pressure of the sensor was fed into a linear recorder. Before the test, the recorder was calibrated with a sufficient amount of distilled water to adjust the number of division intervals for direct measurement of voided urine in milliliters. This allowed calculation of the time point of voiding from the chart speed.

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C.1.2.2

Saluretic Activity in Rats

PURPOSE AND RATIONALE

Excretion of electrolytes is as important as the excretion of water for treatment of peripheral edema and ascites in congestive heart failure as well as for treatment of hypertension. Potassium loss has to be avoided. As a consequence, saluretic drugs and potassium-sparing diuretics were developed. The diuresis test in rats was modified in such a way that potassium and chloride as well as osmolality are determined in addition to water and sodium. Ratios between electrolytes can be calculated indicating carbonic anhydrase inhibition or a potassium sparing effect.

PROCEDURE

Male Wistar rats weighing 100–200 g fed with standard diet (Altromin pellets) and water ad libitum are used. Fifteen hours prior to the test, food but not water is withdrawn. Test compounds are applied in a dose of 50 mg/kg orally in 0.5 ml/100 g body weight starch suspension. Three animals are placed in one metabolic cage provided with a wire mesh bottom and a funnel to collect the urine. Two groups of 3 animals are used for each dose of a test drug. Urine excretion is registered every hour up to 5 h. The 5-h urine is analyzed by flame photometry for sodium and potassium and argentometrically by potentiometrical end point titration (Chloride-Titrator Aminco) for chloride. To evaluate compounds with prolonged effects the 24 h urine is collected and analyzed. Furosemide (25 mg/kg p.o.), hydrochlorothiazide (25 mg/kg p.o.), triamterene (50 mg/kg p.o.), or amiloride (50 mg/kg p.o.) are used as standards.

EVALUATION

- The sum of Na⁺ and Cl⁻ excretion is calculated as parameter for saluretic activity.

- The ratio Na⁺/K⁺ is calculated for natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect. Ratios greater than 10.0 indicate a potassium-sparing effect.
- The ratio

$$\frac{\text{Cl}^-}{\text{Na}^+ + \text{K}^+}$$

(ion quotient) is calculated to estimate carbonic anhydrase inhibition.

- Carbonic anhydrase inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios slight to strong carbonic anhydrase inhibition can be assumed.

MODIFICATIONS OF THE METHOD

Adrenalectomized rats treated with DOCA or aldosterone can be utilized to test **aldosterone antagonists**. Spironolactone has no effect in the absence of a mineralocorticoid, but reverses in a dose-related manner the effect of DOCA on the Na⁺/K⁺ ratio in the urine (Kagawa et al. 1957; Bicking et al. 1965).

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C.1.2.3

Diuretic and Saluretic Activity in Dogs

PURPOSE AND RATIONALE

Dogs have been extensively used to study renal physiology and the action of diuretics. Renal physiology of the dog is claimed to be closer to man than that of rats. Oral absorbability of diuretic substances can appropriately be studied in dogs. Using catheters, interval collections of urine can be made with more reliability than in rats. Simultaneously, blood samples can be withdrawn to study pharmacokinetics.

PROCEDURE

Beagle dogs of either sex have to undergo intensive training to be accustomed to accept gavage feeding

and hourly catheterization without any resistance. The dogs are placed in metabolic cages. At least 4 dogs are used as controls receiving water only, as standard controls (1 g/kg urea p.o. or 5 mg/kg furosemide p.o.) or the test drug group. Twenty-four hours prior to the experiment food but not water is withheld. On the morning of the experiment, the urine bladder is emptied with a plastic catheter. The dogs receive 20 ml/kg body weight water by gavage, followed by hourly doses of 4 ml/kg body weight drinking water. The bladder is catheterized twice in an interval of 1 h and the urine collected for analysis of initial values. Then, the test compound or the standard is applied either orally or intravenously. Hourly catheterization is repeated over the next 6 h. Without further water dosage the animals are placed in metabolic cages overnight. Twenty-four hours after dosage of the test compound, the dogs are catheterized once more and this urine together with the urine collected over night in the metabolic cage registered. All urine samples are analyzed by flame photometry for sodium and potassium and by argentometry (Chloride Titrator Aminco) for chloride content. Furthermore, osmolality is measured with an Osmometer.

EVALUATION

Urine volume, electrolyte concentrations and osmolality are averaged for each group. The values are plotted against time to allow comparison with pretreatment values as well as with water controls and standards. The non-parametric U-test is used for statistical analysis.

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C.2

Assessment of Renal Function

C.2.1

In Vitro Methods

C.2.1.1

Patch Clamp Technique in Kidney Cells²

PURPOSE AND RATIONALE

In the different parts of the kidney (proximal tubules, distal tubules, collecting ducts) fluid is reabsorbed and substances may be transported either from the tubule lumen to the blood side (reabsorption) or vice versa (secretion). Besides active transport and coupled transport systems, ion channels play an important role in the function of kidney cells. The various modes of the patch clamp technique (cell-attached, cell-excised, whole-cell mode) (Neher and Sakmann 1976; Hamill et al. 1981) allow the investigation of ion channels. In addition, the investigation of other electrogenic transport mechanisms, such as the sodium-coupled alanine transport can be studied.

PROCEDURE

The patch clamp technique can be applied to cultured kidney cells (Merot et al. 1988), to freshly isolated kidney cells (Hoyer and Gögelein 1991) or to cells of isolated perfused kidney tubules (Gögelein and Greger 1984). The latter method shall be described in more detail.

Segments of late superficial proximal tubules of rabbit kidney are dissected and perfused from one end with a perfusion system (Burg et al. 1966; Greger and Hampel 1981). The non-cannulated end of the tubule is freely accessible to a patch pipette. Under optical control (differential interference contrast optics with 400× magnification) the patch pipette can be moved through the open end into the tubule lumen and is brought in contact with the brush border membrane. After slight suction of the patch electrode, gigaseals form instantaneously and single potassium or sodium channels can be recorded in the cell-attached or inside-out cell-excised mode (Gögelein and Greger 1984; Gögelein and Greger 1986a).

In order to obtain exposed lateral cell membranes suitable to the application of the patch clamp method, pieces of the tubule are torn off by means of a glass pipette (diameter about 40 µm). As to facilitate the tearing off, the tubules are incubated for about 5 min in

²Contribution by H. Gögelein.

0.5 g/l collagenase (Sigma, C 2139) at room temperature. After tearing off part of the cannulated tubule, clean lateral cell membranes are exposed at the non-cannulated end. The patch pipette can be moved to the lateral cell membrane and giga-seals can be obtained. It was possible, to investigate potassium channels (Gögelein and Greger 1987) and nonselective cation channels (Gögelein and Greger 1986b) in these membranes.

As cells are still part of an epithelial layer and, therefore, are intracellularly coupled, the whole-cell technique is not appropriate in this preparation. On the other hand, cotransport systems can only be investigated by the whole-cell method because the transport rate of a single event is much too small to be resolved in a similar manner as single ion channel events. Consequently, cells of rabbit proximal tubules are isolated as described in detail elsewhere (Hoyer and Gögelein 1991; Heidrich and Dew 1977). After cervical dislocation the kidneys are rapidly excised and placed in ice-cold solution [mmol/l]: 150 K-cyclamate, 10 HEPES, 1 CaCl₂, 1 MgCl₂, pH 7.4. The following steps are performed on ice: After decapsulation, superficial cortical slices of about 0.5 mm thickness are dissected and minced with a scalpel. The tissue is homogenized in a Dounce homogenizer by three strokes with a loose-fitting pestle. The homogenate is then poured through graded sieves (250, 75 and 40 µm) to obtain a population of single cells. Since the predominant tubule section of the cortex of the rabbit kidney is the pars convoluta of the proximal tubule, it can be concluded that the majority of the isolated cells in the cell suspension are of proximal tubule origin. By light microscopy cells are identified by long microvilli distributed over the entire cell surface and can easily be discriminated from remaining erythrocytes, cell detritus and tubular fragments.

By application of the whole-cell mode of the patch clamp technique to freshly isolated cells of convoluted proximal tubules, the sodium-alanine cotransport system could be investigated in detail (Hoyer and Gögelein 1991).

EVALUATION

In isolated perfused renal tubules, concentration response curves of drugs which inhibit ion channels can be obtained with the patch clamp technique. In isolated cells of the proximal tubule, the whole-cell mode of the patch clamp technique enables the investigation of the sodium-alanine cotransport system. The apparent K_m values for sodium and L-alanine can be recorded.

MODIFICATIONS OF THE METHOD

Schlatter (1993) recorded membrane voltages of macula densa cells with the fast or slow whole-cell patch-clamp method. The effects of diuretics and the conductance properties of these cells were examined.

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G.2.1.2

Perfusion of Isolated Kidney Tubules³

PURPOSE AND RATIONALE

The various tubule segments: proximal tubule (PT, S1–S3); descending thin limb of the loop of Henle (DTL); ascending thin limb of the loop of Henle (ATL); thick ascending limb of the loop of Henle (TAL); distal convoluted tubule (DCT); connecting tubule (CNT); cortical collecting duct (CCD); medullary collecting duct (MCD); papillary collecting duct (PCD) have different functional properties. The *in*

³Contribution by R. Greger (first edition) and M. Bleich (second edition).

vitro perfusion of isolated tubule segments (Burg et al. 1966) is the method of choice if one has to identify the site and the mechanism of action of a pharmacological agent which has been shown to act on kidney function in clearance and micropuncture studies.

PROCEDURE

After its invention by Burg et al. (1966) this technique has been used successfully in the kidney tubule segments of several species: man; rabbit; rat; mouse; hamster; snake; birds etc. The tubule segments are dissected from thin kidney slices (<1 mm thickness). Usually dissection can be done using sharpened forceps or needles without the addition of proteases (collagenase). The segment is identified by its anatomical location and by its appearance. A 20–50× lens is used for dissection. Dark field illumination is helpful for the identification of the segment under study. PT, TAL, DCT, CNT, CCD, MCD can all be dissected quite easily. The dissection of DTL, ATL and PCD is much more difficult because these segments are damaged easily by the mechanical dissection. Dissection is usually performed at 4°C in a Ringer type solution.

The dissected segment is transferred into the perfusion chamber by a transfer pipette. The perfusion chamber is mounted in the stage of an inverted microscope (20–400×). The chamber is usually kept at 37°C, and the bath perfusate is also preheated to this temperature. The bath perfusate will depend on the tubule segment under study. In most instances it will contain HCO_3^- and will be bubbled with CO_2 . The metabolic substrate will be acetate for PT and D-glucose for TAL, CCD etc. The actual perfusion is performed with two sets of concentric glass pipettes, one set at the perfusion end; and one at the collection end of the segment. These pipettes are manufactured with special glass forges. The most refined one has been designed by Hampel and Greger. The glass tube is rotated at approximately 1 rps, is moved in perpendicular direction by a remote control, and the heating filament is moved in xy direction also by a remote control. The shaping of the glass is observed continuously by a lens (5–50×). The pulling force is provided by weights fixed on the lower end of the glass tube. The pipettes are cut either by a diamond or by the pulling force of a small glass bead, fixed on the edge of a vertical platinum filament and melted sidewise on the pipette. When the heating current of the platinum filament is switched off the filament retracts and brakes the pipette at the desired site. Greger and Hampel (1981) have modified the original perfusion system of Burg and co-workers. Their device is optimized inasmuch as it guarantees con-

centric alignment of the various pipettes. The forward and backward movement is controlled by small electric motors. At the perfusion side they use 4 concentric pipettes. The outermost one contains sylgard and is driven over the perfused end of the tubule in order to seal this end. The tubule is held by a holding pipette with appropriate dimensions. The tubule is sucked into this pipette up to the constriction. Then the perfusion pipette with a tip diameter smaller than the inner diameter of the perfused segment is advanced into the segment held by the holding pipette. The perfusion pipette is put under hydrostatic pressure of a few to 100 cm to achieve a perfusion rate of 1–20 nl/min. Usually the collapsed tubule lumen opens when the perfusion pipette is advanced. The pipette is advanced in the lumen until it reaches an area of the segment where it appears intact by inspection (200–400×). Within its lumen the perfusion pipette contains yet another pipette, the fluid exchange pipette. With this pipette the composition of the perfusate can be replaced very rapidly (Greger and Schlatter 1983). The collection end of the tubule segment is sucked into a holding pipette. A sylgard pipette is advanced to seal the collection site. The holding pipette at the collection site will contain mineral oil in flux measurements. Then a collection pipette is advanced through the oil to quantitatively collect the perfusate delivered by the tubule.

The **measured parameters** can be as follows:

Flux measurements (Schafer et al. 1974). The collection rate (V_c , nl/min) can be measured by the constant bore collection pipette by timed collections. Radioactive tracers can be added to the lumen or bath fluid. For instance, radioactively labelled inulin can be added to the perfusate (In_p) and can be used to measure volume absorption ($\Delta V = \text{perfusion rate} (V_i - V_c)$). Unidirectional fluxes, bath to lumen and lumen to bath, for any given substance can be quantified, and permeabilities (P_x) can be determined:

$$P_x = (V_i - V_c)L^{-1}[\ln(x_p In_c x_c^{-1} In_p) + 1]$$

where L is the length of the segment; x_p and x_c are the concentrations of x in the perfusate and in the collected fluid; and In_c is the inulin concentration in the collected fluid. Net fluxes of x can be determined as the difference of the unidirectional fluxes or by the chemical determination of Δx (perfusate–collected fluid). This requires very sensitive methods. Electron probe analysis of Na^+ , K^+ , Ca^{2+} , Mg^{2+} etc. has been used to determine the net transport of these ions in various tubule segments (Wittner et al. 1988). Flux studies are usually performed at low luminal perfusion rates of a few nl/min. Substances under study can be added

to the luminal and bath perfusate, and paired data can be obtained under control and experimental conditions (Burg and Green 1973; Stoner et al. 1974; Burg 1980; Burg and Stoner 1976; Dillingham et al. 1993).

Transepithelial electrical measurements. The perfusion pipette can be connected to the high impedance input of an electrometer. The voltage is referenced to the grounded bath. The connections are usually made with agar bridges (80 g agar in 1 l Ringer's solution), and appropriate corrections for liquid junction voltages must be applied. With identical solutions in the bath and in the lumen and with high luminal perfusion rates (> 10 nl/min), any transepithelial voltage (V_{te}) must be caused by active transport = active transport potential (Frömter 1984). Hence, the effectiveness of putative inhibitors of active transport can also be examined by the measurement of V_{te} . According to Ohm's law the determination of the flux of ions also requires the measurement of transepithelial resistance. Greger (1981) has introduced a method which utilizes a dual channel perfusion pipette, made of Q-shaped glass. One channel is used for perfusion and the other for current (I_{te}) injection. The current is defined by a resistor chosen such that the deflection in V_{te} generated by this pulse is in the order of 10–20 mV. Transepithelial resistance (R_{te}) can now be calculated from ΔV_{te} and I_{te} . The ratio of V_{te} and R_{te} is called equivalent short circuit current. It is directly proportional to active transport (Greger 1985). The measurement of V_{te} and R_{te} is much more efficient than flux studies for pharmacological screening, provided that the process under study produces a transepithelial voltage. Several substances can be examined in one single tubule in strictly paired fashion (Schlatter et al. 1983; Wangemann et al. 1986). The time resolution of the measurements is on the order of 1 s, whereas that of flux studies is several minutes at best.

Intracellular electrical measurements. Greger and Schlatter (1983) have developed a method for the use of impalement techniques in the isolated perfused tubule. Very fine tip microelectrodes ($\varnothing < 100$ nm) are used to impale the tubule cell across the basolateral membrane. The actual impalement is performed by a piezo stepper which accelerates the microelectrode to high speed, which makes it possible to penetrate the rigid basal membrane. The simultaneous measurement of V_{te} , R_{te} , and basolateral membrane voltage (V_{bl}) allows for a complete analysis of voltages and resistances (Greger 1985; Ullrich and Greger 1985). Ion selective microelectrodes can also be used in impalement studies, and the cytosolic ion activities for e. g. Na^+ , K^+ , Cl^- can also be determined (Greger 1985). These

methods are all rather difficult to perform. They are of high relevance for the understanding of the function of a given tubule segment and for the detailed description of the mechanism of action of a drug, which, in preceding studies has been shown to act in a given tubule segment.

Patch clamp studies. The combination of *in vitro* perfusion of renal tubules and patch clamp analysis of ion channels in the luminal and basolateral membranes is described in Sect. C.2.1.1.

Fluorescent dyes in the isolated perfused tubule. Several fluorescent dyes for the monitoring of Na^+ , K^+ , Cl^- , Ca^{2+} , pH have become available during the past few years. These dyes can be used in the *in vitro* perfused tubule (Nitschke et al. 1991). The inverted microscope is equipped with an appropriate illumination and filter wheel for excitation. The emission is measured by photon counting or by a video camera. When compared with impalement methods, these techniques are probably easier for routine use.

EVALUATION

For each of the above protocols paired measurements of one or several given parameters of tubule transport are obtained under control conditions and in the presence of a substance under study. Also concentration response curves can be obtained in one single preparation (Schlatter et al. 1983; Wangemann et al. 1986; Wittner et al. 1987). Intracellular measurements are usually required to define the mechanism of action (Greger 1985). Especially the electrical and optical measurements have a very high reproducibility. For screening usually 3 preparations are sufficient. Approximately 10 preparations are required for concentration response curves.

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C.2.1.3

Isolated Perfused Kidney

PURPOSE AND RATIONALE

Isolated kidney is a good tool for studying proximal tubule, but of limited value for distal tubule function. The kidney can be perfused *in situ* and isolated *in vitro*. The isolated kidney can be perfused by a pump using blood or plasma-like solutions. One specific problem of the blood-perfused dog kidney *in vitro* is its instability. After only 1 h of perfusion, glomerular filtration and renal blood flow decline markedly. It was reported that *in situ*-perfused isolated dog kidney seems to be more stable. In isolated perfused rat kidney plasma-like solutions are used for perfusion. This system, by inclusion of a dialyzing unit, provides optimal conditions for maintaining a constant electrolyte composi-

tion of the perfusate. However, function of distal tubule is also grossly impaired in this rat model. The isolated kidney does not acidify tubular fluid, and the concentrating ability is reduced.

PROCEDURE

Kidneys are obtained from anaesthetized male rats with a body weight of 300 to 400 g. The donor animals are fasted overnight prior to surgery, but have free access to water. After the abdominal cavity is exposed by a ventricular incision, the right ureter is cannulated with PE-50 polyethylene tubing and heparin is injected into the vena cava (500 U/kg body weight). The venous cannula is introduced into the vena cava below the right renal vein. The right kidney is freed from the perirenal fat, not disrupting the renal capsule. The renal artery is cannulated via the superior mesenteric artery without interruption of flow. Thereafter, the kidney is continuously perfused with a perfusion solution fed from the gravity system situated 130 cm above the cannula. Ligatures around the renal artery and vena cava above the renal pedicle are tied. The kidney is then removed from the animal and placed in a Plexiglas chamber. A perfusion pressure of 80–90 mm Hg in the renal artery is maintained by adjusting the speed of the perfusion pump. For more details see references.

EVALUATION

After the equilibration period, clearance periods of 20 min are used. Urine samples are collected and perfusate is obtained at midpoint of the clearance period for the evaluation of overall kidney function. For determination of glomerular filtration rate (GFR) and fluid transport, 3H -labelled polyethylene glycol is added to a modified Krebs-Henseleit bicarbonate buffer. Electrolytes are determined in urine by standard flame photometry. Fractional excretions of water, electrolytes and test compounds are calculated.

LIMITATIONS OF THE METHOD

Isolated perfused dog kidneys have been reported to be less stable than those of other species, with glomerular filtration and renal flow markedly decreasing after only one hour of perfusion. The *in situ*-perfused isolated dog kidney seems to be more stable. Distal tubule functions are impaired in isolated perfused kidneys in all species. Urine acidification, concentration and dilution functions are also abnormal, and effects on these cannot be assessed in these models (Kirkpatrick and Gandolfi 2005).

MODIFICATIONS OF THE METHOD

Tarako et al. (1991) evaluated oxygen supply and energy state in the isolated perfused rat kidney.

Metabolic activities of the isolated perfused rat kidney were described by Nishiitsutsuji-Uwo et al. (1967).

Cox et al. (1990) used the isolated perfused rat kidney as a tool in the investigation of renal handling and effects of nonsteroidal anti-inflammatory drugs.

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C.2.2

In Vivo Methods

C.2.2.1

Clearance Methods

PURPOSE AND RATIONALE

Investigations of clearance represent indirect methods for the evaluation of renal function and provide information on the site of action of diuretics and other pharmacological agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the concentration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convolution, would augment the clearance of solute-free water (C_{H_2O}) during

water diuresis and the reabsorption of solute-free water (T_{CH_2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both C_{H_2O} and T_{CH_2O} . On the other hand, drugs that act only in the distal tubule would reduce C_{H_2O} but not T_{CH_2O} .

PROCEDURE

Clearance experiments are performed either in conscious or anaesthetized beagle dogs under conditions of water diuresis and hydropenia. The status of water diuresis and hydropenia may be accomplished as described by Suki et al. (1965). Water diuresis is induced by oral administration of 50 ml of water per kg body weight and maintained by continuous infusion into jugular vein of 2.5% glucose solution and 0.58% NaCl solution at 0.5 ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter. Blood samples are obtained in the middle of each clearance period. After the control period, compounds to be tested are administered and further clearance tests are performed.

Hydropenia is induced by withdrawing the drinking water 48 h before experiment. On the day before the experiment 0.5 U/kg body weight of vasopressin in oil is injected intramuscularly. On the day of the experiment 20 mU/kg vasopressin is injected i.v., followed by infusion of 50 mU/kg per hour vasopressin. To accomplish constant urine flow 5% NaCl solution is infused at 1 ml/min per kg body weight up to i.v. administration of a compound to be tested, followed by i.v. infusion of 0.9% NaCl solution at a rate equal to the urine flow. Glomerular filtration rate (GFR) and renal plasma flow (RPF) are measured by the clearance of inulin and para-aminohippurate, respectively. Therefore, appropriate infusion of inulin (bolus of 0.08 g/kg followed by infusion of 1.5 mg/kg per min) and para-aminohippurate (bolus 0.04 g/kg followed by infusion of 0.3 mg/kg per min) are initiated. Inulin and para-aminohippurate are measured according to Walser et al. (1955) and Smith and al (1945), respectively.

Tracers are administered intravenously to achieve near steady-state concentrations; a priming dose loads the plasma and extracellular compartments, and subsequent infusion replaces renal losses. Once steady state plasma tracer levels are approached, a series of timed urine collections (clearance periods) are performed, with blood samples collected at the either the mid-point or beginning and end of the clearance periods. The urine and blood (plasma or serum) samples are analyzed for tracer(s) and the test compound.

Tracers for the determination of GFR must be freely filtered and then neither secreted nor reabsorbed. This allows the assumption that the amount of plasma cleared of the tracer per unit time represents that which has been filtered through the glomeruli (i.e., GFR). The fructose polysaccharide, inulin (mw ~5200) is the most commonly used tracer in all species and serves as the 'gold standard' to which others are compared (Finco 1983; Ragan and Weller, 1999). Other indicators include isotopes of vitamin B₁₂, sodium iothalamate, iohexol and radiolabelled metal chelates of ethylenediaminetetraacetate (EDTA) and diethylaminotriaminepentaacetate (DPTA) (Sarkar et al. 1988, 1991; Gaspari et al. 1997; Ragan and Weller 1999).

Tracers for the assessment of renal plasma flow must be completely cleared (combination of filtration and efficient tubular secretion to the urine) on first pass through the kidney. This allows the assumption that the volume of plasma cleared per unit time represents that which was either filtered by the glomeruli or bypassed the glomeruli and perfused the tubules. *p*-aminohippurate (PAH) is used for the assessment of RPF, because it is both freely filtered by the glomeruli and actively secreted by the organic acid transport pathway of the proximal tubule. First-pass PAH extraction by the kidneys varies from about 70% to 90% in rats, dogs, and humans (Brenner et al. 1976), but for the purpose of estimating RBF it is assumed to be 100%. Using this assumption, RPF is always slightly underestimated. Tetraethylammonium bromide (TEA), a substrate for the renal cation transporter, may also be used, and is subject to the same limitations (Ragan and Weller 1999).

Inulin is measured colorimetrically, either by acid hydrolysis to generate a green product, or by a series of enzymatic reactions based on inulinase with subsequent reduction of NADH. HPLC methods are used for the remaining exogenous GFR tracers. PAH and TEA are measured colorimetrically (Newman and Price 1999).

EVALUATION

Renal clearance (Cl) of any compound (X) can be determined by comparing the urinary excretion rate of compound X to the plasma concentration of compound X.

The urinary excretion rate is calculated as:

$$\text{Urinary excretion rate (mg/min)} = U_x(\text{mg/ml}) \times V(\text{ml/min})$$

Where U_x represents the concentration of substance X in urine (in mg/ml) and V represents the volume of urine collected per unit time (in ml/min). Thus, the clearance equation may be constructed:

$$\text{Cl}_x(\text{ml/minute}) = U_x(\text{mg/ml}) \times V(\text{ml/min}) / P_x(\text{mg/ml})$$

Where P_x is the concentration of compound X in plasma (in mg/ml)

GFR is estimated by calculating the clearance of the tracer or endogenous substance. RPF estimated using PAH clearance is often designated effective renal plasma flow (ERPF). Renal plasma flow is converted to renal blood flow (RBF) by dividing ERPF by the plasma fraction of whole blood, as estimated from the hematocrit (Hct):

$$\text{RBF} = \text{ERPF} / (1 - \text{Hct})$$

The clearances of other compounds can be compared with inulin clearances to determine how the kidney functions in the elimination of the test compound. A clearance ratio is constructed by dividing the renal clearance of the test compound (X) by the renal clearance of inulin:

$$\text{Clearance Ratio} = \text{Cl}_x, (\text{ml/min}) / \text{Cl}_{\text{inulin}}, (\text{ml/min})$$

A clearance ratio < 1.0 indicates reabsorption of the test substance following filtration, whereas active secretion will result in a clearance ratio of > 1.0.

MODIFICATIONS OF THE METHOD

Endogenous compounds can administered exogenously in place of tracer substances. This has been done for the dog using creatinine (Sapirstein et al. 1955; Finco et al. 2001) and for the rat using cystatin C (Tenstad et al. 1996). Either urine or plasma clearance methods can be used for the former. For the latter, plasma clearance was used to estimate GFR due to the fact that cystatin C is reabsorbed and degraded by the proximal tubule and does not appear in the urine.

Rönnhedh et al. (1996) described a simple method to perform serial renal clearance studies without urine collection in rats. This was applied to non-radiolabeled para-aminohippurate sodium and iothalamate sodium which were used respectively to estimate renal blood flow and glomerular filtration rate.

Gabel et al. (1996) described fast and accurate assays for measuring glomerular filtration rate and effective renal blood flow in conscious rats. An enzymatic method was developed for the determination of inulin

and a colorimetric method was developed for determination of p-aminohippurate in the plasma and urine of rats.

Hropot et al. (1985) described clearance methods in monkeys. Chimpanzees weighing 30.7 ± 10.6 kg were anesthetized with 1 mg/kg Sernylan i.m. Food was withdrawn 24 h prior to the experiment and the animals received only tap water ad libitum. In the morning before the experiment, the urinary bladder of the animals was emptied by catheterization. The urine was discarded. To determine the glomerular filtration rate (inulin clearance), a bolus injection of 50 mg/kg inulin i.v. was given and followed by a continuous infusion of 3 ml/min inulin dissolved in Ringer lactate solution. After an equilibrium of 60 min, urine and blood samples were collected for two control clearance periods of 30 min each. The control periods were followed by intravenous administration of the test preparation in a dose of 20 mg/kg. Thereafter, urine and blood samples were collected during 6 clearance periods. The following parameters were determined: urine excretion, inulin clearance and urate clearance [ml/kg/min], fractional excretion of urate and plasma urate [mmol/l].

Tanaka et al. (1990) evaluated uricosuric and diuretic properties of diuretic agents using clearance studies in urate-loaded dogs and urate-loaded rabbits.

LIMITATIONS OF THE METHOD

First-pass extraction of PAH is highly variable both between species and between individuals within a species, which adds to the inherent inaccuracy of the estimate of RBF by this method. Furthermore, the test compound may interfere with the extraction of either PAH or TEA by competing for transport by the organic anion or cation transporters (Newman and Price, 1999; Ragan and Weller, 1999).

The limitations of the Jaffe method for creatinine determination have been discussed in section 1.1.1. Exogenous creatinine clearance compensates for the insensitivity of the method as well as the interference by endogenous chromagens by artificially increasing the plasma creatinine concentration (Finco 1997).

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C.2.2.2

Assessment of Glomerular Filtration Rate (GFR) and Renal Blood Flow (RBF)

C.2.2.2.1

Assessment of GFR by Plasma Chemistry

PURPOSE AND RATIONALE

Estimation of the glomerular filtration rate (GFR) is considered a sensitive index of functional nephron

mass (Newman and Price, 1999). Point measurements of the plasma levels of several endogenous small molecules (urea, creatinine, 2-(α -mannopyranosyl)-L-tryptophan) or small (less than 66 kDa) proteins (cystatin C (γ -trace), prostaglandin D synthase (β -trace protein), α_1 -microglobulin, β_2 -microglobulin, and retinol binding protein) have been used to assess GFR in many species.

PROCEDURE

Serum or plasma samples are collected from the test animals (if plasma is used, blood should be collected in heparinized tubes). Assay methods vary, and are outlined below:

Urea is most commonly assayed by combined urease methods, in which the urea is first converted to two ammonium ions. The ammonium generated is then measured by either enzymatic or chemical methods. Urea nitrogen values determined by this method (mg/ml) are converted to urea values by the use of appropriate factors (2.14 for urea in mg/ml, 0.357 for urea in mmol/L) (Emeigh Hart and Kinter 2005).

Creatinine is most commonly measured by the Jaffe reaction of creatinine with picrate to generate an orange chromogen. Several enzymatic assays (based on reactions with creatinase or creatinine deaminase) have also been developed. These are equal in sensitivity to the Jaffe method but are considered less likely to be subject to interference by endogenous or exogenous chromogens (Finco et al. 1995; Finco 1997; Newman and Price, 1999). Chromatography (HPLC or LC-MS/MS) assay methods are more sensitive than the Jaffe method and are not subject to interference by endogenous chromogens (Dunn et al. 2004; Owen and Keevil, 2007).

2-(α -mannopyranosyl)-L-tryptophan (MPT) is measured by HPLC (Takahira et al. 2001). Cystatin C assays are all antibody based (nephelometric, agglutination or sandwich ELISA) (Pergande and Jung, 1993; Finney et al. 1997; Jensen et al. 2001), and have been used successfully in dogs, rats, mice and cats (Hakansson et al. 1996; Boekenkamp et al. 2001; Braun et al. 2002; Martin et al. 2002). The other small molecular weight proteins are detected by immunoassays as well.

EVALUATION

Analyte levels are compared either to those of a concomitant control group (using appropriate statistical methods of the group size is large enough) or to laboratory-specific reference intervals for the species, strain, age and sex in question. Elevation of analyte levels outside of the reference range indicate a decrease in GFR,

with the magnitude of the elevation being roughly proportional to the degree of the decrement.

Additionally, algorithms can be used to convert plasma creatinine to creatinine clearance, which provides a reasonably accurate estimate of GFR in humans (Cockcroft and Gault, 1976):

$$\text{GFR} \approx (140 - \text{age}) \times \text{weight (kg)} \times K72 \\ \times \text{Serum creatinine (mg/dL)}$$

Where $K = 0.85$ for women, 1.00 for men

A similar algorithm has been generated for dogs of either gender (Finco et al., 1995):

$$\text{GFR} \approx 2.6 / \text{Serum creatinine (mg/dL)}$$

LIMITATIONS OF THE METHOD

Effects on prerenal (dehydration, blood loss, altered vasomotor tone, age-related decreases in renal blood flow in rats) and/or postrenal factors (obstruction or extravasation of urine to the peritoneal cavity) may cause elevations of the commonly measured analytes that do not reflect effects on the GFR or loss of functional nephron mass (Baum et al. 1975; Corman and Michel, 1987; Finco, 1997; Newmann and Price, 1999).

Urea and creatinine elevations in plasma are in general not sensitive enough to detect low-level alterations (less than 75% loss) of GFR, due to the contribution of renal secretion and/or reabsorption to their overall excretion, (which can compensate for their decreased filtration), to wide variations in baseline levels of some analytes, and to inherent imprecision in the assays used (Finn and Porter, 1998; Price, 2002; Starr et al. 2002; Shemesh et al. 1985). In particular, urea will underestimate GFR (due to extensive tubular reabsorption with decreased GFR) (Baum et al., 1975; Kaplan and Kohn, 1992; Newman and Price, 1999), and creatinine tends to overestimate GFR (because it is secreted by the tubule in many species and secretion increases with reduced GFR) (Shemesh et al. 1985; Andreev et al. 1999; Newman and Price, 1999; Star et al. 2002). In addition, creatinine synthesis is regulated by feedback inhibition which limits the degree of elevation than can occur in plasma (Watson et al. 2002).

The Jaffe reaction for creatinine is subject to interference by numerous endogenous substrates and drugs or compounds (Schwendenwein and Gabler, 2001; Sonntag and Scholer, 2001; Dunn et al. 2004). This effect can be minimized by using appropriate substrate extraction or by the use of kinetic assessments. The urease assay is specific for urea, but increased circulating ammonia (such as occurs in aged plasma samples,

metabolic disorders and portocaval shunting) will react with the subsequent reaction and result in falsely elevated plasma levels (Newman and Price, 1999).

Baseline levels of urea and creatinine are variable, as they are subject to influences on their extrarenal synthesis and release. Plasma urea reflects hepatic synthesis rate and will be elevated with increased protein catabolism (increased dietary protein intake, gastrointestinal hemorrhage, fever, severe burns, corticosteroid administration, sustained exercise or muscle wasting), and decreased with low or poor quality protein diets, modest food restriction in rodents or hepatic insufficiency (Pickering and Pickering, 1984; Finco 1997; Hamberg, 1997; Tauson and Wamberg, 1998; Newman and Price, 1999). Baseline creatinine reflects muscle catabolism and will be elevated in individuals with higher muscle mass or following sustained exercise or acute muscle damage; it will be lower in individuals with loss of muscle mass (Finco, 1997; Newman and Price, 1999). Drugs which compete with creatinine for renal excretion may falsely elevate plasma levels in the absence of renal injury (Andeev et al. 1999).

2-(α -mannopyranosyl)-L-tryptophan (MPT) appears to be less likely to be affected by muscle mass than creatinine. Point-in-time measurements of this tryptophan glycoconjugate correlated extremely well with the inulin clearance, suggesting it may be a superior indicator of GFR. However, the renal handling of MPT has not been examined to determine if plasma levels may be influenced by either reabsorption or secretion (Horiuchi et al. 1994; Gutsche et al. 1999; Takahira et al. 2001).

Pitt et al. (2006) reported the pharmacological profile and toxicity of fluorescein-labelled sinistrin, a novel marker for GFR measurements.

Cystatin C does not appear to be a sensitive index of GFR in the cat (Martin et al. 2002).

The other small protein markers of GFR are detected by immunoassays with reagents specific for the human proteins; the cross-reactivity of these reagents with other species and the usefulness of these markers in animal models have not been well established (Loeb, 1998).

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C.2.2.2.2

Assessment of RBF by Intravascular Doppler Flow Probes

PURPOSE AND RATIONALE

Probes utilizing electromagnetic or Doppler technology may be positioned around or within a renal artery to allow direct measurement of renal blood flow (Yagil 1990; Haywood et al. 1981). Detection of blood flow using Doppler systems is based on changes in the emitted ultrasonic frequency, a Doppler shift, caused by reflection of the signal of moving blood cells. The Doppler shift is proportional to the velocity of blood flow, as indicated by the following equation:

$$\Delta f = \frac{2f_0 v \cos \theta}{c}$$

Where Δf = Doppler peak frequency shift, f_0 = transmission frequency, v = instantaneous peak velocity, θ = angle of incidence of the beam to the bloodstream (assumed to be 0° for linear probes) and c = speed of sound in blood (1570 m/sec) (Chilian et al. 1982).

This technique is particularly useful when rapid or continuous assessment of effects on renal blood flow or assessment of renal vasoreactivity need to be assessed.

PROCEDURE

Doppler probes have been applied to rats, rabbits, cats, dogs, pigs, monkeys and humans; these must be appropriately calibrated prior to experimental use. Animals are anesthetized and the probes are placed within the renal artery via the abdominal aorta, which may be accessed via the femoral or carotid arteries. The diameter of the renal artery at the placement site of the Doppler probe must be determined simultaneously to correct velocity measurements for determination of renal blood flow (see Evaluation).

EVALUATION

A number of parameters can be derived from the Doppler measurements, most commonly including average peak velocity (APV), pulsatility index (PI) and resistive index (RI). Renal blood flow (in mL/min) can be calculated as follows:

$$\text{RBF} = \frac{\text{APV} \times \pi \times \text{D}^2 \times 60}{4}$$

Where D = renal arterial diameter (Doucette et al. 1992).

Resistive index (RI) has been shown in several species to be positively correlated with tubular dysfunction or postrenal obstruction and its return to normal may serve as a prognostic indicator of resolution of tubular disease (Rivers et al. 1997; Shokeir et al. 1996; Tsuji and Taira, 2001).

LIMITATIONS OF THE METHOD

Doppler probe assessment of velocity is accurate primarily in straight blood vessels of small diameter (<4.76 mm) and at relatively low flow rates (<200 mL/min), both of which may be exceeded in normal renal arteries in many species (Lerman and Rodriguez-Porcel, 2001).

In dogs, acute, severe normovolaemic anaemia significantly altered renal artery Doppler parameters without having an influence on Doppler assessment of splanchnic blood flow; the technique may thus be inaccurate in anemic animals (Koma et al., 2006). Furthermore, it is invasive and the size of the test species to which it can be applied depends on the availability of appropriately sized and calibrated probes (Lerman and Rodriguez-Porcel 2001).

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C.2.2.2.3

Assessment of GFR and RBF by Scintigraphic Imaging

PURPOSE AND RATIONALE

Radioactive indicators (^{99c}Tc -DPTA, ^{113m}In -DPTA, ^{99c}Tc -mercaptoacetyltriglycine) may be used to measure renal blood or plasma flows or glomerular filtration rate (Reba et al. 1968). These techniques require intraarterial or intravenous administration of the tracer followed by monitoring of the amount of tracer in the kidneys with an external gamma camera (Fommei and Volterrani 1995).

In species where extrarenal clearance of tracers used in the determination of GFR or RPF is high or variable, external detection methods may actually be more accurate than plasma clearance methods because the extrarenal component of plasma removal is eliminated (Drost et al. 2003).

PROCEDURE

The selected radiotracer is injected either intravenously or intraarterially. External measurements of radioactivity retained in the kidney are made either at timed intervals (rate of accumulation, or slope method) or at a selected interval, usually corresponding to either first pass or peak level of the tracer (percent accumulation, or integral method) (Kampa et al. 2002).

LIMITATIONS OF THE METHOD

These techniques do not yield absolute flow, but rather flow per unit volume or tissue mass. However, algo-

rithms can be developed in the species of interest to allow conversion of tissue uptake to GFR or RBF expressed in standard units by simultaneous determination of plasma clearance of the tracer in preliminary experiments (Kelleher et al. 1991; Kampa et al. 2003), as the correlations between methods for any given tracer are in general high (Delpassand et al. 2000).

In general, assessment of GFR or RBF by external detection methods is less accurate than assessment by clearance methods, although the correction algorithm used for the external detection method can influence the degree of accuracy achievable (DeSanto et al. 1993; Itoh et al. 2000; Itoh, 2003). Integral methods (calculated based on the percent of the dose accumulated) are in general more accurate because they eliminate variability resulting from variable durations of the uptake phase (Kampa et al. 2003). Manual selection of the region of interest (ROI) for the collection of the scintigraphy data also improves the accuracy (Kampa et al. 2002).

MODIFICATIONS OF THE METHOD

Depending on the tracer used, either GFR or RBF can be estimated from the results. The table below lists different tracers that can be used for the assessment of either GFR or RBF (Emeigh Hart and Kinter, 2005):

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Table 1 Radiopharmaceuticals Used for Estimating

| Glomerular Filtration Rate | Renal Blood or Plasma Flow |
|------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------|
| ³ H-inulin | ³ H-para-aminohippuric acid (PAH) |
| ¹⁴ C-inulin | ¹⁴ C-PAH |
| ¹⁴ C-carboxy inulin | S ^{99m} Tc-hippuran analogs |
| ¹⁴ C-hydroxy-methyl inulin | ^{99m} Tc-iminodiacetic PAH (PAHIDA) |
| ¹³¹ I-chloroiodopropyl inulin | ^{99m} Tc-mercaptoacetyltriglycine (^{99m} Tc-Mag3) |
| ¹³¹ I-propargyl inulin | ^{99m} Tc-mercaptosuccinyltriglycine (^{99m} Tc-MSG3) |
| ¹²⁵ I-diatrizoate | ^{99m} Tc-N,N'bis(mercaptoacetyl)-2,3-diaminopropanoate (CO2-DADS-A) |
| ^{125,131} I-iothalamate (Conray – 60) | ^{125,131} I-iodopyracet (Diodrast) |
| ¹³¹ I-diatrizoate (Hypaque, Renografin) | ¹³¹ I-orthoiodohippurate (Hippuran, OIH) |
| ⁵¹ Cr-, ^{99m} Tc-, ^{111,113m} In-, ¹⁴⁰ La-, ¹⁶⁹ Yb-EDTA | ^{67,68} Ga-N-succinyl desferioxamine |
| ⁵¹ Cr-, ^{99m} Tc-, ^{111,113m} In-, ¹⁴⁰ La-, ¹⁶⁹ Yb-DTPA | ⁹⁷ Ru-ruthenocanyl-glycine (Ruppuran) |
| ^{57,58} Co-hydroxycobalamin | ^{99m} Tc-thiodiglycolic acid |
| ^{57,58} Co-cyanocobalamin | |

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C.2.2.3

Assessment of Renal Tubule Functions

C.2.2.3.1

Urinalysis

PURPOSE AND RATIONALE

More than 99% of the reabsorption of glucose, protein and electrolytes occurs in the proximal tubule. Electrolyte concentrations can be affected by other tubule segments, but even low-level changes in proximal tubular function will be reflected by increased urinary excretion of protein and glucose (Stonard et al. 1987; Finco, 1997; Loeb and Quimby, 1999; Newman and Price, 1999; Aleo et al. 2002). Standard and modified urinalysis techniques can be successfully used to assess the effects of compounds on proximal tubule uptake processes (Katsuno et al. 2007).

PROCEDURE

Collection of a good quality sample from the test species is paramount to obtaining high quality data

from the urinalysis. Samples must be collected in clean containers and must be kept free of contamination from food, drinking water, feces, blood and bacteria. For best results, samples should be analyzed promptly (ideally within one hour) after collection, but where analysis must be delayed, the sample must be protected from chemical degradation and evaporation, best accomplished by using collection containers that are appropriately sized for the species in question, tightly sealing the containers when possible, and keeping the specimen cold (4°C) until analysis. If chilled or frozen, samples must be allowed to slowly equilibrate to room temperature before analysis.

Point-in-time samples can easily be collected from most species, either by free-catch, urethral catheterization, manual compression of the bladder or cystocentesis (withdrawal of urine from directly from the bladder with a needle and syringe). Catheterization may require sedation or anesthesia, especially in females of all species and in male pigs whose urethral recess makes this process difficult (Van Metre and Angelos, 1999). Manual compression of the bladder can also be performed on rodents, and cystocentesis may also be performed by a skilled operator using a small (25 gauge) needle and syringe (Loeb and Quimby, 1999).

Timed urine collections in all species (including large animals) can also be obtained by the use of specially designed metabolism cages. A metabolism cage consists of an animal chamber mounted above an excrement collection system. The animal chamber must be equipped with feeder and waterer units if an animal is to be housed in the metabolism cage for more than a few hours. These need to be sized appropriately to the test species of interest (especially regarding the collection container, which must minimize surface

area available for evaporation) and designed to eliminate contamination of the urine by food, drinking water, or feces. In selecting the size of the collection container, the anticipated urine volume should be considered: a mouse will produce 0.25–1 ml in 24 hours of urine, a rat, 10 ml, a hamster, 5–8 ml, a rabbit, 600 ml and a dog or minipig, 500 ml or more (Loeb, 1998; McClure, 1999; Van Metre and Angelos, 1999). Whatever type of metabolism cage is used, the following general precautions are offered:

1. For studies of >24 hours duration, test animals should be acclimated to the metabolism cage for several days prior to study initiation. During this period, animals should be monitored frequently to insure that they learn to use the feeder and waterer systems properly. Test animals should be maintaining or gaining weight prior to study initiation.
2. Feeder and waterer systems should provide ample food and water to meet the animal's needs for the duration of urine collection and all separator systems should function properly. For chronic studies (>5–7 days duration) it is useful to have a complete exchange of feeder, waterer, and urine/feces collector and separator systems so that soiled units may be rapidly exchanged with clean, dry/filled units at regular intervals.
3. Cages should be decontaminated, cleaned, rinsed with distilled/deionized water, and thoroughly dried prior to use. Surfaces used to collect urine may be siliconized or sprayed with a suitable hydrophobic material (PAM, General Foods) to facilitate urine collection.
4. All surfaces contacting urine should be rinsed with distilled/deionized water or appropriate solvents to collect any residuals at appropriate intervals.
5. To preserve the quality of the specimens during prolonged collection times, the opening of the collection vial needs to be small to prevent evaporation and the vial should be surrounded either with wet ice or frozen cold packs to chill the sample promptly once it is deposited (Loeb, 1998; Loeb and Quimby, 1999). Urine may also be collected under mineral oil to prevent evaporative losses. For very small or antidiuretic animals (eg. hamsters, gerbils) placing the cage over a shallow pan of oil and skim feces from the surface may be necessary while collecting urine with a pipette from under the oil. To maximize the volume of the sample collected in rodents in metabolism cages, food should be withheld (this also reduces the risk of contamination of the sample) and water provided (Lee et al. 1998).

Routine urinalysis consists of visual assessment (color, clarity), volume, specific gravity or osmolality, pH, and quantitative or semiquantitative determination of total protein and glucose and microscopic evaluation of urine sediment (Weingand et al., 1996). Urine constituents can be measured semiquantitatively using commercially available “dipstick” test strips (Chemstrips, Roche Diagnostics or Multistix, Bayer Health Care Diagnostics) which contain reagents for the determination of specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, hemoglobin, nitrite and leukocyte esterases (Newman and Price, 1999). These strips may be read manually or using automated analyzers specific to each product.

If quantitative assessment of urine analytes is required (see under “Evaluation”), either urine volume or urine creatinine concentration must be measured and used to “normalize” the concentration of the measured analyte. This will negate the effects of differences in urine concentration between animals and allow for accurate comparison of the results. Creatinine can be measured by the same methods used for plasma..

EVALUATION

Urine volume, combined with assessment of urine concentration (specific gravity or osmolality) can serve as an index to renal function. With severe acute loss of functional nephron mass, urine output is decreased (oliguria) or absent (anuria), while loss of the ability of the kidney to adequately concentrate urine results in the excretion of large volumes of dilute urine.

The commercially available dipstick reagents do not detect the low levels of glucose found in normal urine and thus positive results indicate significant glucosuria in most species (the exception is the gerbil, where dipstick positive glucosuria is normal) (McClure, 1999). If glucosuria is detected, time-matched plasma glucose levels are needed to rule out that this result has not resulted from an increase in the filtered glucose load. If plasma glucose is normal, the appearance of glucose in the urine may indicate a functional deficit in the proximal tubule (Stonard et al. 1987; Finco 1997; Loeb and Quimby 1999; Newman and Price, 1999; Aleo et al. 2002).

Because of inherent inaccuracies in the dipsticks when applied to animal urine (see below under “Limitations of the Method”), positive results by dipstick for protein must be followed by detailed qualitative and quantitative assessment.

Quantitative assessment of urinary protein excretion is necessary to rule out a contribution from

glomerular malfunction. In general, excretion of markedly elevated levels of protein is indicative of glomerular disease, whereas low-level proteinuria indicates tubular damage or very early/lowgrade glomerular injury (Peterson et al. 1969; Finco, 1997).

A number of different methods have been developed for quantitative assessment, including turbidometric, colorimetric (Biuret and Lowry assays as examples) and dye binding assays. All have their advantages and limitations; in general, biuret assays tend to detect all types of proteins with equal sensitivity but require fairly large sample volumes, turbidometric assays can suffer from lack of precision with variations in urine ionic strength, and dye-based methods may suffer from interference with exogenous and endogenous urine substances. The Folin phenol (Lowry), Coomassie brilliant blue and Ponceau S methods have been recommended as being particularly precise for urine samples (Petersen et al., 1969; Dilena et al. 1983; Finco, 1997; Newman and Price, 1999).

Qualitative assessment (identification of the proteins excreted) is necessary to determine if low-level proteinuria has resulted from glomerular or tubular malfunction. Concomitant elevation of albumin and one or more of the low molecular weight proteins (which are freely filtered by the normal glomerulus) indicates that the proteinuria has resulted from decreased tubular reabsorption of proteins, while albumin elevation alone or concurrently with a high molecular weight protein (normally excluded from the filtrate by the glomerulus) indicates primary glomerular injury (Petersen et al. 1969; Finn and Porter, 1998; Gruder et al. 1998; Umbreit and Wiedemann, 2000). Commonly used filtered low molecular weight proteins include retinol binding protein (Price, 2000, 2002; Aleo et al. 2002, 2003), α_2 - or β_2 -microglobulin (Viau et al. 1986; Loeb, 1998; Finn and Porter, 1998; Price, 2000, 2002) or cystatin C (Finn and Porter, 1998; Herget-Rosenthal et al. 2001; Uchida and Gotoh, 2002).

LIMITATIONS OF THE METHOD

Because the commercially available dipsticks are designed for clinical use, they are inaccurate for a number of parameters in animal urine. The specific gravity reagents are completely inaccurate in all species. Dipsticks which use a glucose oxidase method for urine glucose can show a false positive result in species such as the dog and mouse with high urinary ascorbate levels or in urine contaminated with hypochlorite used as a disinfectant (Finco, 1997; Loeb and Quimby, 1999). For all glucose detection methods, test article

formulations that contain glucose or other metabolizable sugars in quantities, should be avoided, as they may transiently overwhelm tubule reabsorption mechanisms and generate false positive test results. Protein detection is based on a bromphenol blue method that is most sensitive for albumin (Newman and Price, 1999), which means that proteinuria that does not result primarily from an increased albumin excretion may not be detected by this method. Furthermore, since these tests are designed for human urine, false positives are frequent in species like the dog, whose normal urine protein levels are just above the lower limits for humans (Finco, 1997), and dipstick tests are invariably positive in male rats and mice which have normal high proteinuria (Loeb and Quimby, 1999).

The sensitivity of the quantitative protein assays to the protein(s) of interest depends in large measure on which protein is used to generate the standard curve. Albumin is most commonly used because it is the most abundant protein in urine and while it is adequate for most methods (Dilena et al. 1983), it will in general underestimate the abundance of many other proteins of interest in urine (Guder and Hofman, 1992).

Qualitative assessment of urine proteins other than albumin is hampered in many species by the lack of suitable immunoassays; with the exception of cystatin C, commercially available antibodies do not cross react with the animal proteins (Loeb and Quimby, 1999; Uchida and Gotoh, 2002). Assessment of urinary retinol excretion has been shown to be a sensitive index to retinol binding protein excretion in the rat (Aleo et al. 2002, 2003).

MODIFICATIONS OF THE METHOD

Point-in-time samples from laboratory rodents may be obtained by taking advantage of the fact that these animals frequently urinate when they are handled or shortly after being removed from their home cage. A skilled, quick and prepared operator with a ready small container or plain microcapillary tube may be able to obtain a small sample in the first instance (Loeb and Quimby, 1999); in the second, the animal can be placed in a small confined space on plastic food wrap and observed carefully for urination, as it has been shown that most rodents will urinate within 20 minutes after removal from their home cages. The sample thus generated can be collected by micropipette (Kurien and Scofield, 1999).

For repeated point-in-time urine samples from rats over short periods of time (one to two weeks after the surgery), the urethra or the ureter can be cannulated. Cannulated rats can also be used to collect accurate

and complete timed urine samples (Mandavilli et al. 1991; Horst et al. 1988).

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C.2.2.3.2

Electrolyte Excretion

Since one of the kidney's primary functions is maintaining electrolyte and mineral homeostasis in the face of fluctuating dietary intake and body needs, examination of plasma and urine electrolyte levels will provide some insight into renal function. Because of the large functional mass of the kidney, alteration of plasma electrolyte levels are usually not detected until the effect on renal function is significant (pathologic). In contrast, urine electrolyte levels examined with knowledge of plasma levels and dietary intake can serve as an extremely sensitive index to the effect of drugs or chemicals on the functional state of the kidney.

In animal studies the diet can be carefully controlled and thus the intake (and hence the plasma electrolyte levels) can be assumed to be fairly constant. Provided there are no sources of significant electrolyte loss resulting from the experimental manipulations (e. g., vomiting, diarrhea, salivation), urine electrolyte levels will reflect the effects of the compound on either GFR (determines the filtered load) or tubular secretion or reabsorption (determines the final urine electrolyte composition).

C.2.2.3.3

Fractional Excretion Methods

PURPOSE AND RATIONALE

Fractional excretion (FE) is the proportion of the filtered load of any analyte that is excreted from the plasma. If both tubular function and plasma electrolyte values are normal, increases in electrolyte FE values clearly reflect a decrement in GFR. With tubular malfunction, the direction of the change in FE values depends on the net direction of electrolyte transport (i. e.,

FE will increase for electrolytes that are primarily reabsorbed and will decrease for secreted electrolytes) (Finco, 1997; Stockham and Scott, 2002).

PROCEDURE

FE assessments can be performed in any species, as it requires only carefully timed complete urine collections and a concurrent assessment of GFR. FE will be unitless if the urine collection period is expressed in minutes, thus (Finco, 1997):

$$\text{FE} = \left(\begin{array}{l} \text{urine electrolyte concentration (mmol/L)} \\ \times \text{urine (mL/collection period)} \\ / (\text{GFR (mL/min)}) \\ \times \text{plasma electrolyte concentration (mmol/L)} \end{array} \right)$$

To eliminate the need for both complete timed urine collections (difficult to do in most animals) and concurrent assessment of GFR, FE values are usually calculated based on point-in-time urine collections by using creatinine excretion during the same time period as an estimator of GFR (Finco, 1997).

Animals are placed in appropriately-sized metabolism cages for an appropriate period of time to allow collection of an adequate volume of urine. At the midpoint of the collection period a blood sample is obtained under appropriate anesthesia (note: the use of CO₂ will falsely elevate plasma potassium levels and render the method inaccurate) for determination of electrolyte and creatinine levels.

Plasma and urine electrolytes and creatinine are determined by standard methods (Durst and Siggard-Andersen, 1999; Newman and Price, 1999; Scott et al., 1999). FE is calculated from the results as outlined below (Stockham and Scott 2002):

$$\text{FE} = \left(\begin{array}{l} \text{urine electrolyte concentration} \\ \times \text{plasma creatinine concentration urine} \\ / (\text{creatinine concentration}) \\ \times \text{plasma electrolyte concentration} \end{array} \right)$$

LIMITATIONS OF THE METHOD

If the Jaffe method for creatinine is used, the investigator must be aware of potential interference due to endogenous chromogens (the error is magnified in species where these chromogens are present in higher concentration in the plasma than in the urine (dog, mouse) (Finco, 1997; Dunn et al. 2004) or possibly the test compound (Sonntag and Scholer, 2001). Additionally, plasma electrolyte levels may fluctuate as the result of eating or due to diurnal rhythms (Finco,

1997). The impact of inaccuracies can be minimized by consistent timing of urine collection, fasting of animals before and during urine collection and the inclusion of a concurrent untreated (vehicle control) group in all studies.

MODIFICATIONS OF THE METHOD

Although FE of sodium is most commonly used to assess tubular function, FE of magnesium has been shown to be the most sensitive index in detecting low-level tubular injury in humans (Barton et al., 1987; Futrakul et al. 1999; Kang et al. 2000; Oladipo et al. 2003). Increased FE of calcium has also been shown to serve as a sensitive index of effects on renal function unrelated to overt renal injury (Lam and Adelstein 1986; Tuso and Nortman, 1992; Elliott et al. 2000)

FE of urea has been recently shown to be more useful than the FE of sodium in distinguishing between prerenal and renal azotemia in humans (Carvounis et al. 2002) and thus may also be useful in making this distinction in animal models. Changes in the FE of urea also reflect changes in urine flow rates (in general, these values move parallel to each other) and can be used as an estimate of this parameter (Finco, 1997). The methods used to detect urea in urine are the same as those used in serum (Newmann and Price, 1999).

FE of anions (ammonium, bicarbonate) can be used to determine the potential mechanism underlying systemic acid base imbalances (Rothstein et al. 1990; Kim et al. 2001).

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C.2.2.3.4

Quantitative Electrolyte Excretion

PURPOSE AND RATIONALE

Fractional excretion methods are useful in that they can identify alterations in analyte excretion with relative ease, as they require only point-in-time assessment of analyte concentration and don't require complete and timed urine collection. Greater sensitivity, however, is obtained with quantitative evaluation of electrolyte excretion. This method is more difficult, requiring carefully timed, complete urine collection, and because plasma levels are not evaluated there needs to be reasonable assurance that the changes in excretion seen do not result from alteration of the filtered load of the analyte in question (e. g., plasma levels and GFR remain consistent). In animal studies where the diet is carefully controlled these factors are not usually of concern.

PROCEDURE

The method can be used on any species, although rats are most commonly used. Animals are usually fasted but allowed access to water; if anesthetized ani-

mals are used, intravenous administration of balanced electrolyte solutions (e. g., lactated Ringer's solution) or 0.9% NaCl solution can be administered by intravenous infusion to ensure that plasma volume (and thus GFR) and electrolyte levels are maintained.

Urine is collected by any appropriate method over a specified period of time. If possible, the urinary bladder should be emptied before collection of test urine is initiated and at the end of the collection period; this can be accomplished in dogs by catheterization and in rodents by handling or manual expression of the bladder. If metabolism cages are used, special care must be taken to avoid evaporation of urine and contamination of the vials with feces or drinking water, as accurate determination of volume is important. The volume of urine collected is determined gravimetrically, and urine electrolytes are measured by standard methods (Durst and Siggard-Andersen, 1999; Scott et al. 1999).

EVALUATION

The volume of urine collected over the time period is defined as the urine flow rate:

$$V = \text{volume collected/time period} \\ \text{(expressed in mL/min)}$$

and electrolyte excretion ($U_X V$) is defined as:

$$U_X V = \{[(\text{concentration of } X \text{ in urine}) \times V] \\ / \text{body weight in g}\} \\ \times 100 \quad \text{(expressed in mmol/min/100 g)}$$

As a general rule, $U_{\text{Na}} V + U_{\text{K}} V = U_{\text{Cl}} V$. If it does not, the assumption must be made that there are additional anions in the urine. Carbonic anhydrase inhibition (i. e., excess urinary bicarbonate may be the culprit if the ratio:

$$\frac{U_{\text{Cl}} V}{U_{\text{Na}} V + U_{\text{K}} V}$$

is less than 0.8.

Net natriuretic activity can be assessed by evaluation of the ratio:

$$\frac{U_{\text{Na}} V}{U_{\text{K}} V} \cdot$$

Values greater than 2.0 indicate a favorable natriuretic effect, and values greater than 10.0 indicate a potassium-sparing effect.

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C.2.2.3.5

Assessment of Tubular Transport Processes using Transport Substrates and Inhibitors

PURPOSE AND RATIONALE

A number of transporters for organic anions and cations are present on both the luminal and basolateral surfaces of the proximal tubules. These are essential to the secretion and/or reabsorption of a number of endogenous substrates as well as functioning in the elimination of xenobiotics. These transporters frequently function as ion exchangers and their activity is thus bi-directional, so that the net direction of transport (i. e., secretion or reabsorption) for any given substrate depends not only on the numbers and activity of the transporters on either side of the tubule but also on the presence and concentration of competing or co-transported substrates. This feature of tubular transport has been one of the major challenges in the development of uricosuric agents for the treatment of hyperuricemia (Terkeltaub et al. 2006). Compounds that affect either

the expression or activity of transporters, are competitive substrates for them, or act as inhibitors of transport thus may affect not only the clearance of therapeutic agents but also that of endogenous metabolites (Wright and Danzler, 2003).

PROCEDURE

The choice of substrate or inhibitor used depends on the transport process or specific transporter of interest. The table below lists the most commonly used substrates and their transporters (note: most inhibitors act competitively and are themselves substrates for the transporter in question).

Transport studies can be performed in any animal model from which urine and plasma may be collected readily. Inhibitors are administered 30 minutes to 1 hour before the administration of the test substance. Substrates are usually administered intravenously, and are detected in urine and plasma most commonly by colorimetric methods.

EVALUATION

Standard clearance or excretion methods are used to assess the effects.

LIMITATIONS OF THE METHOD

Significant sex differences exist in renal transporter expression and activity in rodents and rabbits (Sekine et

| Compound | Most Common Use | Transporter Affected | Comments |
|-------------------------------------------------|-----------------|---------------------------|----------------------------------------------------------------------------------------------------|
| Tetraethylammonium (TEA) | Substrate | OCT1, OCT2, OCT3 | Most commonly used substrate |
| 1-methyl-4-phenylpyridinium (MPP ⁺) | Substrate | OCT1, OCT2 | Can be used to differentiate OCT3-mediated transport |
| Tributylmethylammonium (TBuMA) | Substrate | OCT3 | Can be used to differentiate OCT3-mediated transport |
| Urate | Substrate | URAT1, OAT1, OAT3 | URAT1 is selective for urate in human kidney but has other OA transport functions in other species |
| <i>p</i> -aminohippurate (PAH) | Substrate | OAT1, OAT2, OAT3(?), OAT4 | Most commonly used substrate. Questionable substrate for OAT3 |
| Estrone sulfate | Substrate | OAT3, OAT4, Oatp1 | Oatps are homologs of bile acid transporters in the liver |
| Lucifer yellow | Substrate | OAT1 | Can be detected spectrofluorometrically |
| Phenolsulfonphthalein (Phenol red) | Substrate | OAT3 | Clearance has traditionally been used as a surrogate for urate transport |
| Probenecid | Inhibitor | OAT1, OAT2, OAT3, URAT1 | Used therapeutically and experimentally |
| Cimetidine | Inhibitor | OAT3 | Used to correct creatinine clearance for the contribution of tubular secretion |
| Benzbromarone | Inhibitor | URAT1 | |

al. 2006). Many of the substrates, and all of the inhibitors, are not specific enough for accurate determination of the exact transporter affected by the test compound.

MODIFICATIONS OF THE METHOD

The listed substrates and inhibitors may also be used in *in vitro* models such as isolated tubules or kidney slices. In the latter model, net transport is usually assessed by measuring either the disappearance of the substrate from the medium or, more commonly, the degree or rate of accumulation of the substrate over time in the tissue slice (Kirkpatrick and Gandolfi, 2005).

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C.2.2.4

Assessment of Renal Concentrating Ability

C.2.2.4.1

Solute-Free Water Excretion and Reabsorption

PURPOSE AND RATIONALE

Investigations of the clearance of solute-free water represent indirect methods for the evaluation of several aspects of renal function and provide information on the site and mechanism of action of agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the con-

centration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convoluted tubule, would augment the clearance of electrolyte-free water (EC_{H_2O}) during water diuresis and the reabsorption of electrolyte-free water (ET_{H_2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both EC_{H_2O} and ET_{H_2O} . On the other hand, drugs that act only in the distal tubule would reduce EC_{H_2O} but not ET_{H_2O} .

PROCEDURE

These tests may be performed in any species from which urine and plasma can be readily collected, although low-level changes in concentrating ability may be more readily manifest in rats than in dogs (Sharrat and Frazer 1963; Osbourne et al. 1983). The general procedure involves initially placing the animals in a metabolism cage with unlimited access to water. After collection of a urine sample (16–24 hours is best), the water is withdrawn and the urine is collected for the next 12–16 hours. The urine specific gravity (or preferentially, urine osmolality) is measured and compared both with the value from the hydrated animal and the mean values from the water-deprived control group (Ragan and Weller, 1999).

Assessment of renal urine diluting ability is more cumbersome but can be accomplished in rats by administration of an oral dose of water by gavage representing 5% of the animal's body weight. The animals are then placed in metabolic cages and the urine is collected every 30 minutes for the next 2 hours (Sharrat and Frazer 1963). In dogs, water diuresis can be induced by oral administration of 50ml of water per kg body weight and maintained by continuous infusion into jugular vein of 2.5% glucose solution and 0.58% NaCl solution at 0.5ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter (Suki et al. 1965). The volume and specific gravity (or osmolality) of the collected urine is measured and the results expressed as the percent of the administered dose of water excreted during the time period. Time-matched plasma samples are collected for the determination of plasma osmolality.

EVALUATION

In addition to gross assessment of renal dilution or concentrating ability (as described above), quantita-

tive evaluation of either osmolar clearance, freewater clearance (C_{H_2O}), or solute-free water clearance (EC_{H_2O}) may add additional sensitivity to the evaluation of these functions.

Osmolar clearance can be calculated as follows:

$$C_{osm} = (U_{osm} \times V) / P_{osm}$$

where C_{osm} = osmolar clearance, U_{osm} = urine osmolality (in mosm/kg water), V = urine flow (measured in ml/min) and P_{osm} = plasma osmolality (measured in mosm/kg water). C_{osm} is expressed in ml/min. C_{osm} less than V indicates excretion of a dilute urine (i. e., excess water is being excreted), but C_{osm} greater than V indicates excretion of concentrated urine (i. e., excretion of excess solute).

Freewater clearance (C_{H_2O}) provides an estimate of the amount of urine being excreted in excess that needed to clear solutes, and is calculated traditionally as follows:

$$C_{H_2O} = V X [(1 - U_{osm}) / P_{osm}]$$

(Wesson and Anslow, 1952)

More accurate assessment of the ability of the kidney to appropriately regulate plasma tonicity and/or respond appropriately to antidiuretic hormone (ADH) can be made by calculation of electrolyte-free water clearance (EC_{H_2O}). This formula takes into account only water needed to excrete excess "effective" osmolytes (in general, monovalent electrolytes and their associated anions) and is calculated as follows:

$$EC_{H_2O} = V X \left\{ 1 - \frac{[Na^+ + K^+]_{urine}}{[Na^+ + K^+]_{plasma}} \right\} \quad (\text{Shoker, 1994})$$

LIMITATIONS OF THE METHOD

In general the sensitivity of either urine concentration or dilution tests to detect the effects of test compounds on renal function is quite low, regardless of species (Ragan and Weller, 1999). Furthermore, these tests (especially the urine dilution test) may be significantly altered by extrarenal effects (e. g., vomiting, diarrhea, delayed GI absorption, altered adrenal cortical function). Calculation of osmolar, freewater and electrolyte-free water clearances require accurate assessment of urine flow rates, which will require accurate and complete collection of all urine produced during the time interval, not always easily accomplished in small animal species. Catheterized models should be utilized in this circumstance. In addition, calculated electrolyte-free water clearances may be influenced by the presence of excess effective osmolytes in

either urine or plasma (e. g., in circumstances of hyperglycemia or metabolic acidosis/alkalosis) and the formula must be corrected to account for these if they are known to be present (Shoker 1994; Nguyen and Kurtz, 2005).

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C.2.2.4.2

Assessment of Medullary Osmolarity and Blood Flow

PURPOSE AND RATIONALE

The ability of the kidney to concentrate urine depends entirely on the maintenance of the renal medullary interstitium in a hypertonic state. Hypertonicity reflects two phenomena; water-uncoupled transport of osmotically active substances (sodium and urea, primarily) into the medullary interstitium, and the trapping of those solutes in the interstitium by the vasa recta countercurrent multiplier system. The hypertonic gradient is created by the active transport of sodium into the interstitium in the ascending loop of Henle, and a combination of passive and facilitated transport of urea located in the collecting duct; the magnitude and effectiveness of the gradient is determined by the rate of plasma flow through the vasa recta (Pallone et al. 2003; Sadowski and Dobrowolski 2003). The method described allows simultaneous assessment of both parameters.

PROCEDURE

Male Wistar rats have been used in these experiments. Under appropriate anesthesia, the femoral artery and vein are cannulated for measurement of aortic blood

pressure and infusion of fluids and test compounds, respectively. The left kidney is exposed and placed in a plastic cup as for micropuncture experiments (see 1.2.1.4.), except that the dorsal surface is placed at the top of the cup, facing the operator. The ureter is catheterized and a platinum-iridium admittance electrode is inserted along the corticopapillary axis from the dorsal surface of the kidney (the probe is sized to ensure correct placement of the recording surfaces within the inner renal medulla). The capsule is incised and a needle laser-Doppler (LD) probe is inserted adjacent to the admittance probe. Animals are maintained in intravenous infusions of balanced electrolyte solution to maintain plasma volume and ensure adequate renal perfusion.

Medullary blood flow (MBF) is assessed by the LD probe, which is connected to a perfusion monitor (Periflux 4001, Perimed). The number and velocity of erythrocytes moving between the two optical fibers of the LD probe is determined. Admittance (the reciprocal of impedance) is measured between the tip of the LD probe and the admittance electrode by a conductance meter connected to both of these, following the application of a measuring current at a frequency of 24 Hz. Following the experiment, the kidney is dissected to verify that the LD and electrode have been correctly placed.

EVALUATION

Medullary blood flow (MBF) is expressed in arbitrary perfusion units (PU), based on the voltage generated by the Doppler flux of the blood cells moving beneath the LD probe (roughly, the product of cell number \times velocity). By definition, a 10V signal from the detector is considered 1000 PU. Admittance (Y) is expressed in millisiemens (mS), and is directly related to the ionic tonicity of the tissue (primarily due to the NaCl content). Effects in treated animals are expressed relative to those in untreated controls. If desired, urine may be collected from the ureteral cannula for excretion determinations.

LIMITATIONS OF THE METHOD

Because MBF is expressed in arbitrary units, only relative changes within a single animal can be detected, thus each animal must serve as its own control (although results between animals in the same study can be compared with the use of a calibration algorithm). Significant tissue damage, resulting in non-functional nephrons, does result from the placement of the equipment and thus the technique can only be applied to the innermost medulla (where the glomeruli originate be-

low the depth of the damaged zone). The admittance recordings reflect only the concentration of NaCl in the medulla and thus changes in tonicity resulting strictly from changes in urea concentration cannot be detected by this method.

MODIFICATION OF THE METHOD

Regional changes in medullary tonicity and the steepness of the osmotic gradient can be detected using a system of three needle electrodes that allows assessment of admittance in the outer and inner medulla separately. This arrangement cannot be coupled with the LD probe because excessive tissue damage would result (Sadowski and Portalska, 1983).

An admittance electrode surrounded by a steel cannula fitted to syringe pumps may be used in place of the needle electrode. This setup allows for direct infusion of test substances into the medulla so that local effects may be monitored (Dobrowolski and Sadowski, 2004).

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C.2.2.5

Micropuncture Techniques in the Rat⁴

PURPOSE AND RATIONALE

Micropuncture techniques have been applied to the direct investigation of the effect of diuretics on single nephron function. The observed changes in tubular fluid reabsorptive rates and electrolyte concentrations can be used to assess the mechanism of action. The rat is the model of choice since proximal and distal tubules as well as collecting ducts are accessible for micropuncture.

⁴Contribution by M. Hropot.

PROCEDURE

Clearance and free-flow micropuncture studies are performed in rats with a body weight of about 250 g, anaesthetized by the intraperitoneal injection of thiopentone (Trapanal 50 mg/kg). The animals are fasted for 16 h before the beginning of the experiment, but have free access to tap water. After anesthesia the animals are placed on a thermostatically heated table. Thereafter rats are tracheotomized and carotid artery and jugular vein are cannulated for blood pressure recording, blood sampling, and for infusion of compounds, respectively. The left kidney is carefully exposed by a flank incision, embedded in a small plastic vessel with cotton wool, and bathed with paraffin oil at 37°C. The ureter is cannulated and rectal temperature monitored continuously. A bolus injection of 75 μ Ci inulin 3 H in 0.7 ml NaCl solution is given, followed by 0.85% NaCl solution at a rate of 2.5 ml/min per 100 g body weight. The sustained infusion delivers 75 μ Ci inulin 3 H per hour. The control puncture of tubules is performed 45 min after beginning of the intravenous infusion. The direct collection of tubular fluid samples from proximal and distal tubules is carried out with glass capillaries of 8 to 10 μ m external diameter using a micromanipulator and microscopic observation. Distal tubules are identified by intravenous injection of lissamine green. The control period is followed by the test period. After an equilibration period of 30 min with the compound to be tested, micropuncture is performed again and tubular fluid is collected. The urethral urine is collected and blood sampling is performed in the middle of each clearance period.

The infusion rate/volume is determined by the size of the animal. Distal collection is done by identification of the perfused segment by its dye content; a second micropipette is then inserted, a distal oil or wax block is placed and the infusion fluid can be collected for subsequent analysis (Knox and Marchand 1976; Ramsey et al. 1996; Lorenz et al. 1999; Wang et al. 1993, 1999).

EVALUATION

The following parameters may be determined: inulin clearance (GFR), single nephron GFR, fractional delivery of water, sodium and potassium in proximal and distal tubules and in urine.

Fluid reabsorption (J_v) is assessed by comparing the inulin concentration in the perfusate to that in the collected sample, thus (Wang et al., 1993):

$$J_v = V_0 - VL,$$

Where VL is the collected fluid volume (normalized for the length of the tubular segment over which it was

collected), and:

$$V_0 = VL(INL/IN_0),$$

Where INL = concentration of inulin in the collected sample, and IN_0 is the inulin concentration in the perfusate.

The net flux of the desired analyte (J_X , where X is the analyte of interest – Na, K, HCO_3 , glucose, etc.), is calculated thus:

$$J_X = V_0[X]_0 - VL[X]_L,$$

Where $[X]_0$ is the concentration of the analyte in the perfusion fluid, and $[X]_L$ is the concentration of the analyte in the collected fluid.

All data are expressed as mean values \pm SEM. Comparison of the effects of compounds to be tested with controls is performed by one way analysis of variance and by Student's *t*-test for paired and unpaired data.

MODIFICATION OF THE METHOD

Inulin conjugated to fluorescein isothiocyanate (FITC) may be used in place of the radiolabelled inulin. The inulin concentration in the sample is assessed by comparing the fluorescence in the sample to a standard curve (Lorenz and Gruenstein, 1999).

This technique can be applied to distal tubules as well as to proximal tubules. Distal tubules are identified at the surface of the isolated kidney following micropuncture and perfusion of a proximal tubule as described above. Once the dye reaches the superficial portion of the distal tubule, it is blocked with oil or wax and accessed both proximally and distally as described above (Levine 1985).

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C.2.2.6

Stop-Flow Technique⁵

PURPOSE AND RATIONALE

This procedure is of considerable value in the localization of transport processes along the length of the nephron. During clamping of the ureter, glomerular filtration is grossly reduced. The contact time for the tubular fluid in the respective nephron segments increases, and the concentration of the constituents of tubular fluid should approximate the static-head situation. After release of the clamp, the rapid passage of the tubular fluid should modify the composition of the fluid only slightly. The first samples should correspond to the distal nephron segment, the latest to glomerular fluid. However, with introduction of the micropuncture technique, the stop-flow method appears less attractive.

PROCEDURE

This method can be performed in different animals during anesthesia and was originally described by Malvin et al. (1958). The ureter of an animal undergoing intense osmotic diuresis is clamped for several minutes allowing a relatively static column of urine to remain in contact with the various tubular segments for longer than the usual periods of time. Thus, the operation of each segment on the tubular fluid is exaggerated. Then the clamp is released, and the urine is sampled sequentially. Small serial samples are collected rapidly, the earliest sample representing fluid which had been in contact with the most distal nephron segment. Substances examined are administered along with inulin before the application of uretral occlusion. However, tubular segments downstream from the proximal segments may modify the tubular fluid during its egress.

EVALUATION

In each sample the concentration of a glomerular marker, such as inulin, and the concentration of the

substance under study are measured. Fractional excretion of the substance and the glomerular marker are plotted versus the cumulative urinary volume.

MODIFICATIONS OF THE METHOD

Shinosaki and Yonetani (1989), Shinosaki et al. (1994) performed stop-flow studies on tubular transport of uric acid in rats treated with pyrazinoic acid, an inhibitor of tubular urate secretion.

Tanaka et al. (1990) used stop-flow experiments to test uricosuric and diuretic activities of new compounds in dogs.

Stop flow techniques can be performed in isolated perfused tubular segments using micropuncture techniques. The proximal and distal micropunctures are performed as described, but instead of immediately collecting fluid from the distal micropipette, pressure is applied equal to that of the perfusion pressure at the proximal pipette, until flow ceases at the infusing pipette. Sampling occurs following a period in which the perfusate remains static in the perfused tubule segment. Care must be taken, especially in mice, to prevent tubular leaking with excessive stop-flow pressures or infusion rates (Schnermann, 1999).

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C.3

Impaired Renal Function

C.3.1

Chronic Renal Failure in the Rat

PURPOSE AND RATIONALE

Chronic renal failure is a frequent pathological condition in man. An animal model as described by Acott

⁵Contribution by M. Hropot.

et al. (1987) is of value to test new diuretics under these conditions.

PROCEDURE

Sprague-Dawley rats weighing 150–200 are anesthetized by i.m. injection of ketamine (40 mg/kg) and droperidol/fentanyl (Inovar) 0.25 mg/kg. Through a 6-cm midline incision in the abdominal wall the small bowel and cecum are lifted and placed on saline-soaked gauze sponges. The exposed right kidney is dissected from the retroperitoneal area and the vascular and ureteric pedicles are ligated with 2–0 silk sutures, transected, and the kidney is removed. The renal artery of the left kidney is dissected into the hilum to expose the three main segmental renal arteries. The kidney is not dissected out of the peritoneum. The anterior caudal branch of the artery is then temporarily ligated to establish the volume of renal tissue supplied. The area of ischemia becomes demarcated within 10–15 s. If this approximates 1/4 to 1/3 of the kidney, a permanent ligature is placed. The viscera are then carefully replaced in the abdomen and peritoneum and linea alba are closed with a continuous suture. The skin is closed with stainless-steel clips.

Blood for serum creatinine is collected by retro-orbital puncture under anesthesia at various time intervals up to 12 months. In association with this, 24-hour urines are collected for measurement of creatinine, protein, and specific gravity.

EVALUATION

Serum creatinine increases up to 500 $\mu\text{M/l}$ after 12 months, whereas creatinine clearance decreases. Significantly increased urine volumes are accompanied by decreased urine specific gravity indicating a decreased concentrating ability. Proteinuria is significantly increased. Terminal uremia occurs after 14–15 months.

CRITICAL ASSESSMENT OF THE METHOD

The method may be used for special pharmacological studies as well as for evaluation of renal toxicity of new chemicals.

MODIFICATIONS OF THE METHOD

Sancho et al. (1989) used a similar procedure in rats ligating two of the three terminal branches of the left renal artery, followed by right nephrectomy.

Freeman (1971) induced azotemia combined with hypothermia in rats by ligation of the urinary bladder at the base.

William et al. (1997) described renal ischemia-reperfusion injury in rats. The animals were anesthetized and subjected to 45 min of bilateral renal occlusion using atraumatic vascular clamps before renal perfusion was reestablished. After various time interval (up to 1 week) blood urea nitrogen, creatinine and myeloperoxidase activity in the kidney were determined. The protective effects of an intracellular adhesion molecule monoclonal antibody were tested.

Ishidoya et al. (1995), Klahr and Morrissey (1997) induced interstitial renal fibrosis by unilateral ureteral obstruction in Sprague Dawley rats and tested the effect of ACE inhibitors and angiotensin II receptor antagonists.

Hartenbower and Coburn (1972) described a method for producing chronic renal insufficiency in the chick. By urethral ligation, the function of one kidney was completely eliminated, and the functional mass of the other was reduced by two-thirds. The method resulted in elevation of plasma concentration of uric acid, the major product of protein catabolism in avian plasma, to levels 2–4 times normal for periods as long as 3 weeks.

Two to 3 weeks old White Leghorn cockerels are anesthetized with ether, and the abdominal feathers are clipped. The chick is placed supine on a small operating board with hips flexed and legs extended over the head. An incision is made along the left side of the abdomen extending into the peritoneal cavity. Self-retaining retractors are used to maintain exposure. The right ureter is identified and ligated just proximal to its junction with the cloaca. The left ureter and renal vein are ligated with a single suture near the middle of the left kidney.

The degree of azotemia is assessed by measuring uric acid levels in blood samples 0.2–0.3 ml, obtained by cardiac puncture at intervals of 2–6 days after surgery.

Uric acid blood levels are compared between operated and sham-operated animals. Histological examination is performed after sacrifice of the animals.

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C.3.2

Chronic Renal Failure After Subtotal (Five-Sixths) Nephrectomy in Rats

PURPOSE AND RATIONALE

Subtotal (five-sixths) nephrectomy in rats has been used by many authors as model for chronic renal failure. Levine et al. (1997) used this model to evaluate the hypothesis that under these conditions endogenous angiotensin II modulates *in vivo* bicarbonate reabsorption (J_{ICO_2}) in distal tubules via H^+ -adenosinetriphosphatase and Na^+H^+ -exchange. Bicarbonate reabsorption (J_{ICO_2}) in distal tubules is significantly increased in five-sixths nephrectomized rats.

PROCEDURE

Surgical Procedure

Male Sprague-Dawley rats weighing 230–280 g are anesthetized with halothane via a face mask. Prior to the first incision, the animal is given 20 ml/kg normal saline subcutaneously. On a heated table, a midline laparotomy is performed 2 cm below the xiphoid bone cartilage and the abdomen is opened. The right kidney is isolated and brought out of the abdomen by grasping the fat at the lower pole of the kidney. This fat is bluntly dissected (sparing the adrenal gland), and three silk 4-0 ligatures are passed under the ureter, renal artery, and renal vein, tying one distally and two proximally. The ureter, renal artery and renal vein are then cut between the ligatures, the proximal ties are cut short, and the distal tie is pulled out, removing the kidney. After the area is checked for bleeding, the left kidney is similarly isolated and brought out of the abdomen. The fat around the kidney is bluntly dissected, avoiding excessive handling of the kidney or damage to the ureter, and the left and right renal poles are removed (two-thirds nephrectomy). The left remnant kidney is then returned to the abdomen and moistened with 5 ml of normal saline. The abdomen is closed with sutures and the skin with autoclips. Sham rats undergo the same procedure, except that the kidneys are only touched by

the instruments. All rats are allowed 13–16 days recovery prior to microperfusion.

Microperfusion Experiments

The rats are anesthetized with 100 mg/kg thiobutabarbital sodium and placed on a heated operating table. After tracheostomy, using a PE-240 tubing, the left carotid artery is cannulated for continuous blood pressure measurement and collection of blood for acid-base and electrolyte analyses while the left jugular vein is cannulated with three lines for infusion of fluid, pentobarbital sodium anesthetic and 10% Lissamine green (Levine et al. 1996). The left kidney is exposed by flank incision, carefully dissected from the adrenal gland, and immobilized in a stainless steel cup covered with mineral oil. The ureter is catheterized with PE-50 tubing to ensure proper urine flow.

To replace surgical fluid losses, the rats are infused at 1% body wt/h for 30 min via the jugular vein with donor plasma from control rats. The animals are then maintained on 0.9% saline at 1% body wt/h for the remainder of the experiment.

Two-loop perfusable surface distal tubules are identified by injecting a bolus of 1% Lissamine green into surface proximal loops and observing its passage through the nephron. The distal tubules are perfused at 15 nl/min with a hypotonic solution containing (in mM) 28 HCO_3^- , 26 Cl, 56 Na, 2 K, 1.8 Ca, 22 urea, and 4 gluconate, as well as 0.05% FD and C green dye no. 3 (Keystone, Chicago, IL) and 0.1% albumin. The perfused bicarbonate load, higher than in free flow, is chosen to more easily reveal the effect of inhibitors. Sample collections are quantitative and timed. A 10-min preperfusion period precedes all collections.

Groups of five-sixths nephrectomized rats are treated with various agents, e.g., angiotensin II, angiotensin₁ receptor antagonists, or Na^+/H^+ antiporter inhibitors.

Analyses

Whole blood and urine pH and P_{CO_2} are measured quantitatively by an electrode blood-gas system and HCO_3^- concentrations are calculated. Plasma and urine Na^+ and K^+ concentrations are measured by flame photometry and Cl^- concentrations by electrotitration. Plasma total protein concentrations and urine specific gravity are measured by refractometry, and hematocrits are determined by microcapillary reader. Urine osmolalities are determined by freezing-point osmometry. Plasma creatinine concentrations are determined by the Jaffé method without deproteinization.

Perfusate and sample total CO₂ concentrations are measured by microcalorimetry (Levine et al. 1996). A standard curve is run before sample analysis, and standard samples bracket the determination of sample and perfusate CO₂ determination.

EVALUATION

The perfusion rate (R_P) is calculated as the product of the measured collection rate (R_C) and the ratio of inulin concentration in collected tubular fluid and perfusate. Water absorption (J_V) is calculated as the difference between the calculated perfusion rate and the measured collection rate (R_P minus R_C). J_{CO_2} is calculated as

$$J_{\text{CO}_2} = [(R_P C_P) - (R_C C_C)]/L$$

whereby C_P and C_C are the measured CO₂ concentrations in perfusate and collected fluid, respectively, and L is the tubular length in millimeters, measured by dissection after latex injection.

Data are expressed as the means \pm SE. Statistical significance is assessed by two-tailed unpaired Student's t -test or one way analysis of variance (ANOVA) followed by either Dunnett's test for multiple comparisons vs. control or the Newman-Keuls test for all pairwise comparisons. Tests indicating a value of $P < 0.05$ indicate a statistically significant difference between groups.

MODIFICATIONS OF THE METHOD

Function of the remnant kidney after subtotal (5/6) nephrectomy in **rats** has been used for many purposes:

- to test the potential benefit of calcium antagonists (Tolins and Raji 1990; Jarusripipat et al. 1992; Van den Branden et al. 1997) or antioxidants (Vaziri et al. 1998),
- to verify the effect of ACE-inhibitors (Pelayo et al. 1990; Kakinuma et al. 1992; Ashab et al. 1995; Liu et al. 1996; Ali et al. 1998; Cohen et al. 1998; MacLaughlin et al. 1998), angiotensin antagonists (Kohzuki et al. 1994, 1995; Brooks et al. 1995; Barreto-Chaves and Mello-Aires 1996; Noda et al. 1997; Lariviere et al. 1998; Rocznik et al. 1999) and endothelin receptor antagonists (Nabokov et al. 1996; Potter et al. 1997; Wolf et al. 1999; Brochu et al. 1999; Shimuzu et al. 1999), or Na⁺/H⁺ antiporter inhibitors (Fernandez et al. 1994),
- to study the influence of hormones, such as parathyroid hormone (Fukagawa et al. 1991; Urena et al. 1994; Yi et al. 1995; Schaefer et al. 1996), growth hormone (Santo et al. 1992; Garcia de Boto et al.

1996), insulin-like growth factor I (Hazel et al. 1994; Mak and Pak 1996; Tonshoff et al. 1997), vasopressin (Bardoux et al. 1999), atrial natriuretic factor (Wong and Wong 1991, 1992), Luk et al. 1995) or erythropoietin (Poux et al. 1995; Zhou et al. 1997), during development of chronic renal failure.

Kimura et al. (1999) reported a model of progressive chronic renal failure in *rats*, produced by a single injection of microspheres (20 to 30 μm in diameter) into the left renal artery after right nephrectomy.

Cowley et al. (1996) described the **Han:SRPD rat** strain which develops autosomal dominant polycystic kidney disease with chronic renal failure that resembles human autosomal dominant polycystic kidney disease.

Chronic renal failure can be induced by feeding a lithium containing diet (40–50 mmol/kg) to newborn *rats* until an age of 55–65 weeks (Christensen et al. 1992, 1997; Nyengaard et al. 1994).

Stockelman et al. (1998) described chronic renal failure in a **mouse** model of human adenine phosphoribosyltransferase deficiency. Hamilton and Cotes (1994) used a partial nephrectomy model in *mice* with two-thirds of total renal mass excised to evaluate erythropoiesis and erythropoietin production from extrarenal sources such as the submandibular salivary gland. Koumegawa et al. (1991) suggested the DBA/2FG-*pcy mouse*, which develops numerous cysts in kidney cortex and medulla, a progressive anemia and an elevation of blood urea nitrogen, as useful spontaneous model of progressive renal failure.

Brown et al. (1990) studied the metabolism of erythropoietin in normal and uremic **rabbits** with 5/6 nephrectomy. Bonilla-Felix used *rabbits* after 75% nephrectomy to study the response of cortical collecting ducts from remnant kidneys to arginine vasopressin.

Fine et al. (1990), Vaneerdeweg et al. (1992) described surgical techniques for kidney resection to produce chronic renal failure in **dogs**.

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C.3.3

Experimental Nephritis

C.3.3.1

General Considerations

Various experimental procedures were described as models for glomerulonephritis in human beings. Most of them were developed in rats and rabbits. They involve the reactions of antibodies against renal components, such as Masugi nephritis (Masugi and Sato 1934), Heymann nephritis (Heymann 1959), nephrotoxic serum nephritis (Unanue and Dixon 1967), crescentic type anti-glomerular membrane nephritis (Nagoe et al. 1994, 1998), anti-Thy1 nephritis (Chen et al. 1999).

Moreover, MRL Mpf lpr/lpr (MRL/lpr)-mice were described which spontaneously develop a severe disease with many symptoms very similar to human systemic lupus erythematoses, i.e. hypergammaglobulinemia, and glomerulonephritis (Theofilopoulos and Dixon 1981; see also I.2.2.16).

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C.3.3.2

Nephrotoxic Serum Nephritis

PURPOSE AND RATIONALE

Nephrotoxic serum nephritis is produced in animals by administration of heterologous antibody against glomerular basement membrane. This is regarded as an experimental model of human glomerular immune injury resulting in glomerulonephritis (Unanue

and Dixon 1967). The glomerular lesions induced by nephrotoxic serum nephritis vary with species. The Wistar–Kyoto rat is susceptible to the induction of a crescentic glomerulonephritis following small doses of nephrotoxic serum (Kushiro et al. 1998; Suzuki et al. 1998).

PROCEDURE

Preparation of Nephrotoxic Serum

Normal Wistar rat kidneys are fully perfused with physiologic saline through a catheter placed in the aorta. Renal cortical tissue is removed, homogenized and diluted with physiological saline at about 20% suspension. Two ml of renal cortical homogenate are emulsified with an equal volume of Freund's complete adjuvant. This emulsion is injected subcutaneously into rabbits twice a month for two months. Seven days after the last injection, the rabbits are bled from the carotid artery under anesthesia. The sera are decomplexed for 30 min at 56°C and absorbed with freshly harvested rat erythrocytes.

Experimental Protocol

Male Wistar–Kyoto rats weighing 150 g receive either continuous administration of the test drug by an osmotic pump (ALZA Co., Palo Alto, USA) or saline. Twenty-four hours later, the rats are injected with 1 ml of nephrotoxic serum. At 9, 12, and 14 days, urine samples are collected and urinary protein levels are measured using the Lowry method. At 14 days the rats are sacrificed under ether anesthesia, and both kidneys are removed. Portions of these tissues are processed for light microscopy, immunofluorescence staining and immunoperoxidase staining.

For light microscopy, tissues are fixed and embedded in paraffin. Sections are stained with hematoxylin and eosin, and periodic acid Schiff's reagent. Twenty glomeruli are examined per rat and number of glomeruli which forms crescent is counted.

Indirect immunofluorescence studies are performed on 3 µm-thick cryostat sections which are air dried and incubated with anti-rat intercellular adhesion molecule-1 (ICAM-1) antibody (Tamani and Miyasaka 1990) for 60 min at room temperature. After washing the antibody binding is visualized by incubating the sections for 30 min with fluorescein isothiocyanate-labeled goat anti-mouse IgG.

Direct immunofluorescence studies are performed on 4 µm thick cryostat sections, which are incubated with fluorescein isothiocyanate-labeled goat anti-rat IgG, goat anti-rat C3, goat anti-rat fibrinogen and goat anti-rabbit IgG.

The staining intensity of 20 glomeruli per rat is semiquantitatively assessed into 4 grades.

For **immunoperoxidase staining** the distribution of leukocytes is examined using an immunoperoxidase ABC kit (Vector Lab, Burlingame, USA). Nonspecific protein binding is blocked by incubating the cryostat sections with 10% bovine serum in Tris-buffered saline for 20 min. Nonspecific staining is blocked by 15 min incubation with avidin and then biotin using the avidin-biotin blocking kit (Vector Lab). Endogenous peroxidase activity is inhibited by incubating the sections in methanol containing 0.3% H₂O₂ for 20 min. Sections are first incubated for 60 min with primary antibodies at room temperature, incubating monoclonal antibodies against rat monocytes/macrophages (ED-1), rat CD4 and rat CD8. Then the sections are incubated with biotinylated donkey anti-mouse IgG for 30 min at room temperature. Biotinylated horseradish peroxidase is applied for 30 min at room temperature. Peroxidase activity is developed in 3,3'-diaminobenzidine and hydrogen peroxide. The sections are then counterstained with Mayer's hematoxylin. The number of ED-1 positive cells, CD4 positive cells and CD8 positive cells per glomerular cross-section is counted in 20 glomeruli per rat.

EVALUATION

All data are expressed as mean ± SEM. Significance of differences between groups is determined using Wilcoxon's test.

MODIFICATIONS OF THE METHOD

Masugi and Sato (1934), Krakower and Greenspon (1951), Heyman et al. (1959, 1965), Eddington et al. (1968) already described experimental allergic glomerulonephritis in rats.

Ito et al. (1983), Nagao et al. (1994, 1998) induced crescentic type antiglomerular basement membrane nephritis in male Sprague Dawley rats by injecting 6.5 mg rabbit gamma-globulin in 0.25 ml Freund's complete adjuvant into the hind foot pads, following the injection of 0.6 ml of rabbit anti-rat glomerular basement membrane serum into the tail vein.

Couser et al. (1978) studied the development of immune deposits on the subepithelial surface of the glomerular capillary wall in isolated rat kidneys perfused at controlled perfusion pressure, pH, temperature, and flow rates with recirculating oxygenated perfusate containing bovine serum albumin in buffer and sheep antibody to rat proximal tubular epithelial cell brush border antigen.

Hayashi et al. (1996) tested the effects of a flavonoid in original-type anti-glomerular basement membrane antibody associated glomerulonephritis in male Sprague Dawley rats on upregulation of intracellular adhesion molecule expression and on increase in leukocyte function-associated antigen positive cells in nephritic glomeruli.

Nagamatsu et al. (1999) found beneficial effects of an angiotensin II type I receptor antagonist in anti-glomerular basement membrane antibody-associated nephritis in rats.

Sanaka et al. (1997) evaluated the effects of a free radical scavenger on the progression of nephrotoxic serum nephritis in male Sprague Dawley rats. The rat glomerular basement membrane was prepared according to the method of Krakower and Greenspon (1951) who localized the nephrotoxic antigen within the isolated renal glomerulus.

Kawasaki et al. (1992) induced crescentic glomerulonephritis with a small dose of nephrotoxic serum in WKY rats, which was characterized by the early infiltration of CD8 positive cells in glomeruli. *In vivo* depletion of CD8 positive cells completely prevented proteinuria and crescent formation.

Okuda et al. (1990) provided evidence of an elevated expression of transforming growth factor- β , proteoglycans and fibronectin in glomerulonephritis induced in rats by injection of anti-thymocyte serum.

Hamada and Nagase (1996), Chen et al. (1999) induced anti-Thy1 nephritis with the antibody to the Thy-1 antigen which is present in the mesangial cells of the glomeruli. The early pathobiological cellular events are characterized by invasion of platelets, polymorphonuclear leukocytes, and monocytes into the glomerulus which occurs within hours after induction of nephritis. Complement-dependent mesangiolytic then ensues between day 1 and 3.

Liebler et al. (2004) induced acute anti-Thy1.1-mesangioproliferative glomerulonephritis by administering 500 μ g of Moab 1-22-3 dissolved in PBS as a single-shot injection into the tail vein of the rats on day 0. Moab 1-22-3 is a monoclonal antibody directed against the Thy1.1-like antigen on the surface of rat mesangial cells.

Passive Heymann nephritis (Heymann 1959) was used a model by Hara et al. (1991), Nagao et al. (1996), Heise et al. (1998). The disease is induced in rats by heterologous antibody to crude renal border antigen Fx1A. The model is characterized by granular deposition of heterologous and homologous antibody and complement along the glomerular capillary wall and as a counterpart, extensive electron-dense subepithe-

lial deposits are seen at the ultrastructural level. Massive proteinuria develops after a latent period of 2 to 4 days in the absence of glomerular hypercellularity.

Kawasaki et al. (1995) studied the therapeutic effect of combined treatment with monoclonal antibodies against intercellular adhesion molecule 1 (ICAM-1) and lymphocyte-function-associated antigen 1 (LFA-1) in **Masugi nephritis** of Wistar-Kyoto rats.

Thaiss et al. (1989) evaluated the effect of the immunosuppressant cyclosporin A on an active model of *in situ* immune complex glomerulonephritis. Wistar rats were preimmunized with human IgG and 2 weeks after the last antigen injection, the left kidney was perfused with cationized human IgG in order to induce unilateral *in situ* immune complex glomerulonephritis.

Okubo et al. (1990) studied the immunosuppressive effects of FK506 on active Heymann's nephritis and the autologous phase of Masugi nephritis.

Rennke et al. (1994) developed a model system of acute nephritis in the rat whereby a chemically reactive form of the hapten azobenzenearsonate is introduced directly in to the left kidney of pre-immunized Brown Norway rats.

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C.3.4

Experimental Nephrosis

PURPOSE AND RATIONALE

Some compounds used in antineoplastic therapy induce nephrosis in animals, like the antibiotic adriamycin (=doxorubicin) (Milner et al. 1991, 1994; Chagnac et al. 1994; Wapstra et al. 1996; De Boer et al. 1999; Mutti et al. 1999) or daunomycin (Kimura et al. 1993), the aminonucleoside puromycin (Yayama et al. 1993; Guoji et al. 1994; Magil 1996; Ebihara et al. 1997; Nosaka et al. 1997; Park et al. 1998; Asami et al. 1999; Pedraza-Chaverri et al. 1999) or cisplatin (Abdel-Gayoum et al. 1999).

These models allow to test the protective effects of drugs even after the renal disease in established, i. e. mimic the clinical situation (Wapstra et al. 1996).

PROCEDURE

Male Wistar rats with an initial weight of about 300 g receive a single intravenous dose of 2 mg/kg adriamycin. Twice a week during a 12-weeks period, the animals are weighed, 24-h urine is collected and blood pressure is measured by the tail-cuff method.

During the first 5 weeks, all animals are kept on a low sodium diet with tap water ad libitum. After stabilization of proteinuria (5 weeks), animals are divided into 2 groups receiving either low sodium or normal sodium diet. After a week stabilization on these diets, animal receive different doses of treatment in their drinking water. These regimen is continued until the end of the study (week 12), at which time all animals are sacrificed and blood samples and kidney tissue are obtained.

During each blood pressure measurement session, five measurements are recorded for each animal. The blood pressure is taken as the mean of the last 3 recordings. Urinary protein is determined by the Pyrogallol Red-molybdate method (RA-1000 Technicon). Urinary sodium, creatinine and urea and serum electrolytes, creatinine, albumin, cholesterol and triacylglycerols are measured by a standard autoanalyser technique. Kidney samples are fixed in paraformaldehyde and embedded in paraffin. Sections

are stained with the periodic acid/Schiff technique. Focal glomerular sclerosis is scored semiquantitatively by light microscopy.

EVALUATION

Analysis of co-variance is used to compare the experimental groups (defined by diet or dose of drug) after 2 weeks of treatment (week 8) and the end of the study (week 12) with the baseline value (week 6) as a co-variable. Tuckey's method is used for comparison of groups receiving different doses of drug. The other statistical comparisons are performed using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Fawn-Hooded rats develop systemic hypertension and spontaneous age-dependent glomerulosclerosis with proteinuria (Mackenzie et al. 1997).

Spontaneous nephrosis with proteinuria occurs in Dahl salt-sensitive rats fed on a normal sodium diet (Yoneda et al. 1998).

Mizuno et al. (1999) studied the ICGN mouse strain as a unique model for naturally occurring nephrotic syndrome.

Focal segmental glomerulosclerosis with heavy proteinuria has been found in mice in which the *Mpv17* gene was inactivated (*Mpv17*^{-/-} mice). Binder et al. (1999) recommended these animals as model of steroid-resistant nephrosis sensitive to radical scavenger therapy.

Kimura et al. (1993) described strain specificity in the susceptibility of mice to daunomycin-induced nephrosis.

Klahr and Morrissey (1997) described the effects of ACE inhibitors and angiotensin II receptor antagonists on various parameters associated with renal interstitial fibrosis induced by unilateral ureteral obstruction in rats.

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C.4 Uricosuric and Hypo-Uricemic Activity

C.4.1 In Vitro Methods

C.4.1.1 Inhibition of Xanthine Oxidase In Vitro Indicating Hypouricemic Activity

PURPOSE AND RATIONALE

Synthesis of uric acid primarily occurs in the liver, but the kidney has an important role in the pathophysiology of hyperuricemic syndromes. Because uric acid is poorly soluble, excessive amounts in the circulation may precipitate out into the tissues, particularly the joints, resulting in a painful arthropathy ("gout"). In humans, this condition is usually the result of faulty tubular transport of urate, resulting in increased net reabsorption. Attempts to treat hyperuricemia with tubular transport inhibitors (theoretically increasing urinary excretion of urate) frequently exacerbate the condition because tubular transport is bi-directional; reduction of net uric acid synthesis by inhibition of xanthine oxidase is the preferred therapeutic approach. Furthermore, in renal hypoxic conditions, xanthine oxidase contributes to renal injury by the generation of oxygen free radicals and xanthine oxidase inhibition has been shown to be useful in such conditions (Shinosaki and Yonetani, 1991; Rhoden et al. 2000; Terkeltaub et al. 2006).

Xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid. Xanthine oxidase is a complex metalloflavoprotein containing one molybdenum, one FAD and two iron-sulfur centers of the ferredoxine type in each of its two independent subunits. Usually, the enzyme is isolated from cow's milk. The enzyme is inhibited by allopurinol and related compounds. The production of uric acid from the substrate (xanthine) can be determined by measuring the change in optical density in the UV range.

PROCEDURE

The test compound is incubated with xanthine oxidase (usually derived from milk, sometimes derived from rat liver or small intestine), EDTA and phosphate buffer solution (pH 7.8) at 37°C. Control solutions without test compound are incubated under identical conditions. Following addition of xanthine, the change in absorbance is determined.

Assay conditions:

- wavelength: 293 nm

- line path: 10 mm
- final volume: 1.0 ml

EVALUATION

The percent inhibition of xanthine oxidase is determined relative to control solutions.

IC_{50} values of test compounds are calculated.

Standard data:

- Allopurinol: IC_{50} : ca 10^{-8} mol/l

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C.4.1.2 Urate Uptake in Brush Border Membrane Vesicles

PURPOSE AND RATIONALE

The urate-anion exchanger system in brush border membrane vesicle, which mediates hydroxyl ion gradient-dependent urate uptake, is the most likely route for the mediation of urate transport in the first step of urate reabsorption in the proximal tubules. Luminal drugs which inhibit urate reabsorption are inhibiting the transport of urate by blocking the urate/anion exchanger.

PROCEDURE

Male Sprague Dawley rats weighing 320–380 g are sacrificed by decapitation and the kidneys are removed immediately. All steps for the preparation of brush border membrane vesicles are carried out at 4°C. Renal cortex is homogenized for 2 min in a medium containing 250 mmol/l mannitol, 10 mmol/l Tris, 16 mmol/l HEPES buffer (pH 7.5) using a Physcotron homogenizer. The homogenate is centrifuged at 2400 g for 10 min and the supernatant is centrifuged at 2800 g for 20 min. Subsequently, the supernatant is discarded and the loosely packed membrane-rich layer is flushed off from the bottom densely packed brown pellet. The membrane-rich layer is resuspended manually

in 250 mmol/l mannitol containing 10 mmol/l Tris-HEPES (pH 7.5) using a Dounce homogenizer and MgSO_4 is added to a final concentration of 10 mmol/l. After standing for 20 min, the suspension is centrifuged at 2400 g for 20 min and the supernatant containing brush border membranes is recentrifuged two more times at 2400 g for 20 min. The final supernatant is centrifuged at 28,000 g for 20 min and the pellet is suspended in a small amount of medium containing 150 mmol/l mannitol, 50 mmol/l potassium phosphate buffer (pH 7.5) and 2 mmol/l MgSO_4 to a final protein concentration of 4–8 mg/ml. The brush border membrane vesicle preparation is frozen and stored at -80°C until use.

After preincubation of the brush border membrane vesicle preparation for 2 h, $[2\text{-}^{14}\text{C}]\text{urate}$ uptake is initiated by adding 200 μl of incubation medium to 20 μl of the membrane suspension. The incubation medium has the following composition (mmol/l): 150 mannitol, 2 MgSO_4 , 50 potassium phosphate buffer, pH 6.0 or 7.5, 0.02 $[2\text{-}^{14}\text{C}]\text{urate}$, and various concentrations of the inhibitor. At 10 s after the addition of the incubation medium, 200 μl portions of the suspension are pipetted onto the center of prewetted cellulose acetate filters kept under suction. The vesicles retaining on the filter are washed immediately with 5 ml of an ice-cold solution containing 150 mmol/l mannitol and 50 mmol/l potassium phosphate buffer, pH 6.0 or 7.5, which is used at the same pH as the incubation medium. Preincubations and incubations are performed at $23 \pm 1^\circ\text{C}$. Each experiment is performed in triplicate. Corrections are made for the radioactivity bound to the filters in the absence of membrane vesicles. The term of the OH^- gradient-dependent urate uptake is defined as the difference between the uptakes in the incubation medium at pH 6.0 and that at pH 7.5. The OH^- gradient-dependent urate uptake at 10 s is assumed to present an initial velocity.

EVALUATION

From a concentration-response curve relating log concentration of drug to the logit activity of the OH^- gradient-dependent urate uptake for 10 s, IC_{50} (concentration producing 50% of inhibition) is determined by least-squares regression analysis.

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C.4.2

In Vivo Methods

C.4.2.1

Diuretic and Uricosuric Activity in Mice

PURPOSE AND RATIONALE

Renal excretion of uric acid consists of three components: complete filterability of uric acid in the glomerulus, subsequent tubular reabsorption, and tubular secretion (Gutman and Yü 1961). Pronounced species differences have been described in uric acid metabolism including man. Mice were recommended for primary screening of uricosuric drugs.

PROCEDURE

Male NMRI mice weighing 25–30 g are used. On the evening prior to the experiment, food but not water is withheld. In the morning, the mice are orally loaded with 50 ml/kg 0.9% NaCl-solution. Together with the sodium load the test compound is applied by gavage in 2% starch suspension. Controls receive saline and starch suspension only. Groups of 5 mice are placed into metabolism cages. Urine is collected over 4 h. In the urine sodium and potassium are determined by flame photometry, chloride by argentometrically with potentiometrical end point titration (Chloride titrator, Aminco), uric acid by the Uriquant-method, creatinine by the Jaffé-reaction, as well as pH and osmolality.

EVALUATION

Urine excretion is calculated in ml/kg. Uric acid-, creatinine- and ion-excretions are calculated in mmol/kg and expressed as percent changes versus controls. The changes are evaluated statistically using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Some saluretic-diuretic agents, like ethacrynic acid, are inactive in the rat, when given orally. Moreover, uricosuric activity in mice is less reliable than that in primates.

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C.4.2.2

Hypouricemic Activity After Allantoxanamide Treatment in Rats

PURPOSE AND RATIONALE

Most species used for pharmacological experiments have rather low blood levels of uric acid. Experimental hyperuricemia can be induced by inhibition of the enzyme uricase. In most species uricase metabolizes uric acid to allantoin. Allantoxanamide blocks uricase and increases endogenously synthesized uric acid. This increase is blocked by compounds like allopurinol.

PROCEDURE

Non fasted male Sprague Dawley rats weighing 230–280 g are treated by intraperitoneal injection of 250 mg/kg allantoxanamide suspended in 5 ml/kg sesame oil. The test compound is applied orally in a dose of 50 mg/kg in 40 ml/kg water. Likewise, the standard compound allopurinol is given in a dose of 50 mg/kg. Eight rats are used for the each dose of test drugs and standard. The animals are placed individually into metabolism cages with free access to food and water. Urine is collected during the periods of 1 to 6 and 7 to 24 h. Blood is withdrawn by retroorbital puncture prior and 2, 6 and 24 h after compound administration. Uric acid is determined with the Uric-aquant method in plasma [mmol/l] and urine [mmol/l].

EVALUATION

Mean values of uric acid concentrations in plasma at the different time intervals and mean values of uric acid excretion after 6 and 24 h of the test group are compared with the control group (allantoxanamide treated only) using Student's *t*-test.

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C.4.2.3

Hypouricemic and Uricosuric Activity After Potassium Oxonate Treatment in Rats

PURPOSE AND RATIONALE

Increase of uric acid in serum of rats is induced by a special diet and the uricase inhibitor potassium oxonate. Uric acid concentration in serum and uric acid excretion in urine prior and after three experimental days are determined.

PROCEDURE

Male Wistar rats weighing 250 g are placed individually in metabolic cages. They are offered a special diet containing 5% fructose, 3% uric acid, 2% potassium oxonate (2,4-dihydroxy-1,3-triazine-6-carboxylic acid) and 0.001% artificial sweetener. Drinking water consists of a 0.5% solution of potassium oxonate. The animals are treated orally with 50 or 100 mg/kg of the test compound or the standard (allopurinol) dissolved in 5 ml/100 g body weight of 0.5% potassium oxonate solution. The treatment is repeated on the second day. On the third day 24 h urine is collected and the animals are sacrificed by exsanguination. Concentrations of uric acid and electrolytes (Na^+ , K^+ , Cl^-) are determined in blood and urine.

EVALUATION

Concentrations of uric acid and electrolytes in blood and urine of animals treated with the test compound are compared statistically with control and standard drug treated animals.

MODIFICATIONS OF THE METHOD

Clearance techniques in oxonate-treated rats were used by Yonetani et al. (1987), Shinosaki et al. (1991), Dan et al. (1994).

Sugino and Shimada (1995) tested uricosuric effects in oxonate-loaded rats, in the pyrazinoic acid suppression test and in the phenolsulphonphthalein test.

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C.4.2.4

Phenol Red Excretion in Rats

PURPOSE AND RATIONALE

Phenol red (= phenolsulfonphthalein) excretion is an indirect test for uricosuric activity. After intravenous injection phenol red is mainly eliminated by active secretion in the proximal tubulus of the kidney. Treatment with uricosuric agents decreases the secretory activity of tubulus cells resulting in a delayed excretion of phenol red. Plasma values of phenol red are increased in treated animals as compared to controls.

PROCEDURE

Male Wistar rats weighing 120–150 g are treated orally with the test compound or the standard 30 min prior to intravenous injection via the tail vein with 2.5 ml/kg of a 3% aqueous solution of phenolsulfonphthalein. For

intravenous application, 5.0 ml/kg of the test drug solution are injected immediately after the phenolsulfonphthalein injection followed by flushing with 2.5 ml/kg saline. By retro-orbital puncture blood samples are withdrawn after 30, 60 and 180 min. Blood (0.2 ml) is diluted with 2 ml 0.9% NaCl-solution and centrifuged. To 1 ml of the supernatant 1 ml of 1% sodium carbonate solution and 8 ml of saline are added. Using a spectrophotometer (Eppendorf, Hamburg) extinction at 546 nm is determined.

EVALUATION

Extinction values are calculated for total blood. At each time interval the values in treated rats are compared statistically with those of controls.

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C.4.2.5

Uricosuric Activity in Dalmatian Dogs

PURPOSE AND RATIONALE

Most species have low plasma levels and low renal excretion of uric acid. The Dalmatian dog is an exception with excessive uric acid excretion, but with relatively high plasma levels. Explanations are a genetically determined defect in tubular reabsorption of the filtered urate (Friedman and Byers 1948; Kessler et al. 1959) and a defective hepatic uricase activity (Yü et al. 1971). The Dalmatian dog is being used for studies of uricosuric agents.

PROCEDURE

Conscious male Dalmatian dogs with a body weight of about 20 kg are used. Food but not water is withheld 24 h prior to the experiment. The animals are placed individually in metabolic cages. The urinary bladder is emptied by catheterization. Twenty ml/kg drinking water is applied by gavage. Every 2 h the animals are catheterized again, blood is withdrawn from a jugular vein and additional 8 ml/kg drinking water is applied by gavage. The urine and blood values obtained after the first 2 h serve as control. Then, the test compound is applied either i.v. or orally. Up to 8 h blood and urine samples are collected every 2 h. No water is given af-

ter the last sampling. The dogs stay over night in the metabolic cages. Twenty-four hours after beginning of the experiment, venous puncture and catheterization is performed once more. Blood and urine samples are analyzed for uric acid (Uricaquant-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

EVALUATION

The values after application of the drug are compared with predrug values.

CRITICAL ASSESSMENT OF THE METHOD

Dalmatian dogs bred by commercial breeders are not always homozygous. Therefore, not every dog is suitable for experiments on uric acid excretion.

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C.4.2.6

Uricosuric Activity in Cebus Monkeys

PURPOSE AND RATIONALE

The Cebus monkey is described not to possess uricase (Simkin 1971) and to have different metabolic conditions for uric acid than other experimental animals. This species is chosen for special studies of anti-uricopathic drugs since it resembles more closely human uric acid metabolism.

PROCEDURE

Cebus monkeys (*Cebus albifrons*) of either sex weighing 3.0 to 5.0 kg are used. Twenty-four h prior to the test, food is withheld, but water is available ad libitum. On the morning of the experiment, the animals receive

20 ml/kg drinking water by gavage, followed by oral application of the test compound. Control animals receive water only. The animals are placed in individual metabolism cages and the spontaneously voided urine is collected after 2, 6, and 24 h. After 2 and 6 h, additional 4 ml/kg water is given by gavage. From a cubital vein blood is withdrawn prior to the experiment and 2, 6 and 24 h after application. Urine and serum samples are analyzed for uric acid (Uricaquant-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

EVALUATION

Allopurinol and probenecide are used as standard drugs and are compared with test compounds.

CRITICAL ASSESSMENT OF THE METHOD

The use of the Cebus monkey as animal model has been proven to be the most valuable method to test putative hypouricemic compounds (Hropot 1988).

MODIFICATIONS OF THE METHOD

Onuma et al. (1988) used Cebus monkeys for evaluation of uricosuric effects of an aryloxyacetic derivative.

Yonetani et al. (1987) performed clearance experiments with uricosuric drugs in anesthetized chimpanzees.

Dan et al. (1989) tested the activity of AA-193, an uricosuric agent in rats, mice and Cebus monkeys.

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C.5 Influence on Lower Urinary Tract

C.5.1 In Vivo Studies

C.5.1.1 Micturition Studies

PURPOSE AND RATIONALE

Urinary incontinence is a major psychosocial, medical, and economic problem. The most common condition to be treated pharmacologically is incontinence due to detrusor instability. The response of the urinary bladder to filling with increasing volumes of fluid (cystometrogram) is a common procedure for evaluating bladder function in both animals and humans. The response of the vesico-urethral complex can be arbitrarily divided into the collection and expulsion phases. The nervous control of the detrusor, the internal and the external sphincter has been reviewed by Kuru (1965). A detailed description of the nervous control of the urinary bladder of the cat has been given by De Groat (1975). The pharmacology of lower urinary tract muscles and penile erectile tissues has been reviewed by Anderson (1993). Ferguson and Christopher (1996) reviewed urine bladder function and drug development. Urine storage and timely expulsion of bladder content are produced through the coordinated activation of a series of reflexes involving cholinergic, sympathetic, and, possibly, purinergic, serotonergic, and peptidergic innervation. In view of this complexity, *in vivo* models were developed for the quantitative analysis of the effects of drugs on the function of the vesico-urethral complex (Maggi et al. 1983, 1985, 1986, 1987a, b, 1992).

PROCEDURE

Male Wistar rats weighing 340–360 g are anesthetized by subcutaneous injection of 1.2 g/kg urethane. The left jugular vein is cannulated for drug injection. Body temperature is kept constant by means of a heating pad maintained at 37°C. Through a midline incision of the abdomen, the urinary bladder is exposed and emptied of urine by application of a slight manual pressure. A 20-gauge needle is inserted through the apex of the bladder dome for 3–4 mm into its lumen. The needle is connected to a pressure transducer by means of a polyethylene tubing (1.5 mm OD and 1.0 mm ID) and the whole system filled with saline. The tubing is provided with an internal coaxial polyethylene tubing (0.6 mm OD and 0.3 mm ID) inserted through a side hole and sealed by a drop of epoxy resin. The second

tubing serves for intravesical infusion of fluid and is connected, through a peristaltic pump, to a saline reservoir.

Intraluminal pressure signals are delivered to an amplifier and displayed on a four channel polygraph. Warm saline-soaked cotton wool swabs are laid around the exteriorized organ to maintain its temperature and to keep it moist in experiments involving the topical application of substances on the bladder dome.

After a 15-min equilibration period at zero volume, variations in intraluminal pressure are recorded in response to continuous infusion of saline at a rate of 2.8 ml/h at 37°C for 30–40 min by means of a peristaltic pump connected to the polyethylene tubing inserted into the bladder. This infusion rate simulates the maximal hourly diuresis within the physiological range. In each preparation the infusion is continued until micturition occurs. Micturition is referred as the emission of several drops of fluid during a sustained phasic contraction of the detrusor muscle which is followed by return to zero or, in any case, to a value lower than that recorded just before micturition.

For both intravenous and topical administration, substances are dissolved in saline.

EVALUATION

In each experiment, the following parameters are evaluated:

1. pressure threshold = intraluminal pressure value recorded just before micturition
2. volume threshold = the volume of infused saline required to obtain micturition
3. maximal amplitude of micturition contraction
4. residual volume after micturition

The effect of substances on the compliance of the bladder wall is evaluated by comparing the volume-pressure-relationship of treated animals with that of controls.

Statistical analysis of the data is performed by means of the Student's *t*-test for paired or unpaired data, or by means of analysis of variance followed by the Tukey test. Statistical analysis of nonparametric data is made by the chi square test.

MODIFICATIONS OF THE METHOD

Either chemical (6-hydroxydopamine, reserpine) or surgical (section of hypogastric nerves) sympathectomy produces a picture of detrusor hyperreflexia and urine dropping, mimicking cystometric finding in human disease (Maggi et al. 1987a).

Postius and Szelenyi (1983) described a model for *in vivo* screening of spasmolytic compounds using the rat bladder.

Dray (1985) used the spontaneous, volume-induced contractions of the urinary bladder in the anesthetized rat to assess the central activity of substances with opioid properties.

Pietra et al. (1990) studied the effects of some antidepressants on the volume-induced reflex contractions of the rat urinary bladder. The urinary bladder of anesthetized rats was filled via the recording catheter by incremental volumes of warmed saline until bladder contractions occurred as a result of central activity. Volume-induced contractions were then recorded and occurred rhythmically and reproducibly for 2–3 h. Drug activity was assessed in each animal against the background frequency of bladder contractions, for a 15-min time period following intravenous administration of different doses.

Harada et al. (1992) proposed a method for rapid evaluation of the efficacy of pharmacologic agents and their analogs in enhancing bladder capacity and reducing the voiding frequency. Conscious rats were placed in a restrainer over a urine collector. The collector was secured to an Statham UC3 strain transducer, the output of which was amplified by a Gould bridge amplifier. Data were monitored on a polygraph. This method has been simplified by the use of an infrared photodiode sensor and matched phototransistor than can detect the appearance of urine flowing from the bottom of a metabolic cage, recording the frequency of these events. This method produces results comparable to the original cup-fore transducer methods (Argentieri and Argentieri 2002).

Conte et al. (1991) proposed a method for simultaneous recording of vesical and the external urethral sphincter pressure in urethane-anesthetized rats.

Angelico et al. (1992) reported *in vivo* effects of different antispasmodic drugs on the rat bladder contractions induced by topically applied KCl.

Oyasu et al. (1994) measured spontaneous bladder contractions caused by raising the intravesical volume in anesthetized rats.

Yaksh et al. (1986) described a chronic model for study of micturition in unanesthetized rats. A bladder catheter was implanted chronically through laparotomy and externalized percutaneously.

Horváth et al. (1994) reported an ultrasonic method to study the influence of drugs on micturition in intact rats.

Tillig and Constantinou (1996) described videomicroscopic imaging of urethral peristaltic function in

anesthetized rats. Cystometrograms were performed by recording continuously the bladder pressures while detecting micturition using a sensor placed at the orifice of the urethra. Renal pelvic pressure was measured during continuous perfusion using a nephrostomy inserted through the parenchyma. A catheter was placed in the femoral vein for intravenous drug administration. The left pyelo-ureteric junction and the upper part of the ureter were visualized using a stereomicroscope equipped with a video camera and a tape recorder. One syringe pump was used for filling the bladder to perform continuous cystometrograms. An other syringe pump was used for infusion of indigo carmine to assist the visualization of the bladder pressure.

Conte et al. (1988) developed a cystometric technique for quantitative studies on physiopharmacology of micturition in conscious, freely moving rats.

Seif et al. (2004) performed urinary bladder volumetry by means of a single retrosymphysically implantable ultrasound unit.

Peterson et al. (1989), Noronha-Blob et al. (1991) described *in vivo* cystometrograms studies in urethane-anesthetized and conscious **guinea pigs**.

Moreau et al. (1983) described simultaneous cystometry and uroflowmetry for evaluation of the caudal part of the urinary tract in **dogs**.

Imagawa et al. (1989) reported an *in vivo* procedure for functional evaluation of sympathetically mediated responses in lower urinary tract of dogs.

Häbler et al. (1990, 1992) examined the functional properties of unmyelinated and myelinated primary afferent neurons innervating the pelvic viscera in anesthetized **cats**. The axons were isolated from the intact dorsal root and the intact or chronically de-efferented ventral root of segment S2. The responses of the neurons were studied with natural stimulation of the urinary bladder using innocuous or noxious increases of intravesical pressure.

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C.5.2

Studies in Isolated Organs

C.5.2.1

Studies on Renal Pelvis

PURPOSE AND RATIONALE

Periodic contraction of the renal pelvic wall is important to the process of concentration of urine in the renal medulla as well as the propulsion of urine from the kidney into the bladder (Santicioli and Maggi, 1997, 1998; Knepper et al. 2003). The isolated renal pelvis of the guinea pig has been used to examine the effects of compounds on this activity. (Maggi and Giuliani 1991, 1992; Maggi et al. 1992a, b, 1992c, 1994, 1995; Giuliani and Maggi 1996; Santicioli et al. 1995, 1997; Patlachini et al. 1998; Bigoni et al. 1999).

PROCEDURE

Male albino guinea pigs weighing 250–300 g are used. Following appropriate anesthesia and euthanasia, one whole kidney and attached ureter are removed and placed in oxygenated Krebs solution. The renal pelvis is carefully dissected from the renal parenchyma, separated from the ureter, cut and connected to threads

to record motility along the circular axis. The preparation is suspended in a 5 ml organ bath and mechanical activity recorded by means of an isotonic transducer (load 1 mN). Transmural electrical field stimulation is made by means of platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S 88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) are delivered in trains of 10 s duration at frequencies of 5 to 10 Hz.

Experiments commence after a 60 to 90 min equilibrium period after which amplitude and frequency of spontaneous activity has reached a steady state. *In vitro* capsaicin desensitization is made by exposure of the preparation to 10 μ M for 15 min, followed by washing out and further equilibration for 30–60 min.

Concentration response curves to noradrenaline and acetylcholine are performed by non-cumulative addition to the bath at 20 min intervals. Contact time of drugs is 15 min.

EVALUATION

The amplitude and frequency of spontaneous contractions are assessed and the Motility Index (MI) is calculated as follows:

$$MI = (\Sigma amp/5) \times F$$

where $\Sigma amp/5$ is the mean amplitude of five contractions (in mN) and F is the frequency (in min^{-1}) of those five contractions. Concentration-response curves are generated by plotting the concentration versus the MI (either raw or expressed as a percent of control, using the parallel incubation as the control) (Davidson and Lang, 2000).

MODIFICATIONS OF THE METHOD

Zhang and Lang (1994), Lang et al. (1995), Lang and Zhang (1996) Teele and Lang (1998) recommended circumferentially cut strips from the proximal renal pelvis of guinea pigs since these strips contract more frequently than strips cut from the mid region.

Kimoto and Constantinou (1990, 1991) studied contractility of smooth muscle strips from the pacemaker regions and pelviureteric junction of renal pelvis from **rabbits**.

Kondo et al. (1992) determined the effects of dobutamine and terbutaline on adenylate cyclase activity and cyclic AMP content in the renal pelvis of rabbits.

Seki and Suzuki (1990) made intracellular recordings to study the electrical properties of smooth muscle cells in the rabbit renal pelvis.

Zwergel et al. (1991) developed an intact **canine** model to measure renal pelvic pressure after complete

ureteral obstruction with a balloon catheter inflated in the distal ureter.

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C.5.2.2

Propagation of Impulses in the Guinea Pig Ureter

PURPOSE AND RATIONALE

The mammalian ureter provides one of the clearest examples of electromechanical coupling in smooth muscle. The ureter smooth muscles are electrically and mechanically quiescent but, when depolarized to threshold, they fire an action potential characterized by an unusually long-lasting potential and generate a phasic contraction (Shuba 1977; Brading et al. 1983; Meini et al. 1995). Both the action potential and the accompanying contraction critically depend upon the influx of extracellular calcium through voltage-sensitive L-type channels, which are enhanced and blocked by dihydropyridine drugs, Bay K 8644 and nifedipine, respectively. The model predicts that suppression of action potentials at any site of the ureter will suppress the propagation of contraction and peristalsis. (Weiss 1992).

PROCEDURE

A number of species (most commonly rats and guinea pigs) have been used for whole-mount or strip preparations. Following appropriate anesthesia, the whole kidney and ureter are excised and placed in a Petri dish

containing oxygenated Krebs solution for dissection. A 4–5 cm long piece of ureter is dissected from the inferior renal pole and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex partitions are used to separate the renal-, middle- and bladder-sites. They include a window covered with condom rubber: a small hole (about 300 μm) is made in the rubber to enable the passage of the ureter. Proximal to each partition, the renal and bladder ends are pinned to a Sylgard support. The distal portions of the renal and bladder ends are connected via a pulley to isotonic transducers (Basile 7006, load 2 mN) for recording of mechanical activity on a two-channel polygraph. Each compartment is perfused by means of a peristaltic pump at a rate of 1 ml/min with oxygenated Krebs solution at 34°C.

The frequency and amplitude of spontaneous contractions are recorded. Alternatively, electrical field stimulation is applied to either compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter. Square wave pulses (5–25 ms pulse width, 20 V) are automatically delivered every 100 s by means of a GRASS S88 stimulator.

Drugs are applied by superfusion at the middle site. Amplitudes of contraction are recorded.

EVALUATION

The amplitude and frequency of contractions are assessed and the Motility Index (MI) is calculated as follows:

$$MI = (\Sigma \text{amp}/5) \times F$$

where $\Sigma \text{amp}/5$ is the mean amplitude of five contractions (in mN) and F is the frequency (in min^{-1}) of those five contractions.

Data are expressed as a percent of the control value and appropriate statistical analysis is performed. Concentration-response curves may be plotted and pA_2 or pD_2 values may be calculated. Hill coefficients and EC_{50} values may also be calculated (Weiss et al. 2002).

MODIFICATIONS OF THE METHOD

Ureter preparations have been made from pig or human tissues collected at slaughter or following surgery, respectively. Ideally, no more than 20 minutes should elapse between tissue collection and incubation. Tissues are placed into cold (4°C) Krebs buffer or suitable balanced electrolyte solution and cut into rings of 0.5 to 1 cm in length. The rings are attached to a force transducer, suspended in an organ bath at 37°C, and

allowed to equilibrate for up to 1.5 hours while the frequency and amplitude of spontaneous contraction is measured. If the preparation demonstrates acceptable and stable frequency and amplitude of spontaneous contraction within this time period it is considered suitable for experimental use. In pigs, the washout period between treatments is variable and thus the preparation is monitored for return to pre-treatment baseline before the next treatment is applied. In human tissue, spontaneous contraction seldom occurs and electrical stimulation (trains of 300 ms at an interval of 200 s and impulses of 200 mA with a duration of 6 ms at a frequency of 50 Hz) is required. A washout period of 20 minutes between treatments is used (Weiss et al. 2002).

The evaluation of regional differences in response, and the effect of regional responses on adjacent regions, can be evaluated in a whole ureter preparation where each region remains physically attached but can be isolated for the application of test compounds or stimuli. The entire ureter is dissected and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex partitions are used to separate the proximal (renal), middle, and distal (bladder) ends. They include a window covered with condom rubber: a small hole (about 300µm) is made in the rubber to enable the passage of the ureter. The proximal portions of each segment are pinned to a Sylgard support, and the distal portions are connected to isotonic transducers for recording of mechanical activity. Each compartment is perfused separately and electrical field stimulation can be applied to any compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter (Meini et al. 1995).

Tomiyama et al. (2003) characterized functional muscarinic cholinergic receptors in the isolated ureter of dogs.

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C.5.2.3

Studies on Urinary Bladder and Internal Urethral Sphincter

PURPOSE AND RATIONALE

Several authors investigated the influence of drugs on isolated parts of the lower urinary tract. Ueda et al. (1984) studied the effects on smooth muscle of the rabbit bladder dome, trigone and proximal urethra.

PROCEDURE

Male New Zealand rabbits weighing 2–3 kg are sacrificed and the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue is removed, the bladder and the urethra are dissected into dome, trigone and proximal urethral preparations. All strips are cut transversely being approximately 2 × 6 mm unstretched. Ligatures are placed on both ends of the strips and one end is attached to a tissue holder and the other to a strain gauge force-displacement transducer connected to a polygraph on which isometric tension changes are recorded. Each of the strips is then placed into a 20 ml tissue bath containing Krebs-Ringer solution bubbled with 95% O₂+5% CO₂ at 37°C. Resting tension is adjusted to 1 g during an equilibrium period of at least 2 h. The contractile and relaxant responses are measured as increases or decreases from the resting tension. Dose-response curves are performed in a cumulative manner. Tissues are pretreated with an antagonist 20 min before the addition of an agonist.

EVALUATION

The values are expressed or plotted as the means ± SE and pA₂ values are calculated according to Arunlakshana and Schild (1959). Data are analyzed using the *t*-test, analysis of variance, Dunnett's test and regression analysis.

MODIFICATIONS OF THE METHOD

Rats and guinea pigs have been used for whole bladder preparations. Following appropriate anesthesia, the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue is removed, the preparation is placed *en bloc* into appropriate bal-

anced electrolyte solution (Tyrode's or Krebs buffer) bubbled with 5% CO₂ and 95% O₂. The urethra is cannulated and connected to a pressure transducer, and the bladder is filled with a volume of solution adequate to evoke a contractile response. The bladder preparation is allowed to equilibrate for 30 minutes before any experimental manipulations are performed. The amplitude and frequency of pressure changes are recorded (Birder et al. 1999; Drake et al. 2003).

Alternatively, contractility can be assessed visually. Surface blood vessels or externally applied markings (India ink or carbon particles) are used as landmarks. Video image recording of the bladder preparations are made during the experimental manipulations. Still images from the video are evaluated and the change in linear distance between pairs of landmarks is used as an index of contractility (Drake et al. 2003).

Pietra et al. (1990) studied the effects of some antidepressants by the *in vitro* inhibition of carbachol-induced contractions of rat detrusor strip preparations. The detrusor muscle tissue (bladder dome) was cut in a semicircular direction and further dissected into strip preparations measuring approximately 2 × 20 mm.

The preparation of the isolated, innervated urinary bladder of the rat was reported by Hukovic et al. (1965). Electrical stimulation was performed by a bipolar electrode from the nerves running close to the ureter.

Mapp et al. (1990) used the isolated rat urinary bladder to study the pharmacological modulation of the contractile response to toluene diisocyanate.

Maggi et al. (1985) used isolated detrusor strips of rat bladder connected to an isometric strain gauge and stimulated by field stimulation.

Anderson (1978) recommended the **rabbit** detrusor muscle as a unique *in vitro* smooth muscle preparation. Rabbit detrusor muscles are thin and devoid of underlying submucosal tissue with parallel fiber orientation. The tissue exhibits autorhythmicity, characteristic of most single unit type smooth muscle preparations and can be employed in either isometric or isotonic organ bath recording systems.

Honda and Nakagawa (1986) studied the effects of the optical isomers of an alpha-1 adrenoceptor antagonist in rabbit lower urinary tract and prostate.

Khanna et al. (1977, 1981) evaluated the *in vitro* responses of three segments of rabbit lower urinary tract, e. g., the bladder body, the bladder base and the proximal urethra.

Ferguson and Marchant (1995) studied the inhibitory actions of GABA on rabbit urinary bladder muscle strips.

Andersson et al. (1983) studied the electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. In rabbits, two circular transverse sections, each 4 mm long, were taken from the middle and upper parts of the urethra. Human urethral preparations were obtained from male patients undergoing cysto-urethrectomy en bloc because of bladder cancer. Rings of tissue were taken from the membranous and supra- and infra-collicular parts of the prostatic urethra.

Andersson et al. (1992) used transversal strips from the middle and upper part of rabbit urethra to study the involvement of nitric oxide in the electrically-induced, nerve-mediated relaxation.

Contractile responses to nervous stimulation can be assessed in whole isolated bladder preparations. The pudendal nerve remnants can be identified near the ureter and can be attached to electrodes for stimulation of the bladder. Alterations in the contractile responses by the test compound are considered to result from effects at the neuromuscular junction (Hukovic et al. 1965; Weetman, 1972; Dhattiwala and Dave, 1975).

Inci et al. (2003) used a coaxial bioassay system to test the effect of inflammation on rat urinary bladder-dependent relaxation. In this bioassay model, the bladder was the donor organ for the assays and rat anococcygeus muscle was the assay tissue. The rat bladders removed from control or treatment groups were used in its original shape after making small cuts at bladder neck and dome. The rat anococcygeus muscle was always isolated from control group of rats. The anococcygeus muscle was placed in the lumen of the bladder, so that freely enveloped by the bladder, and mounted under a resting tension of 1 g in a 20 ml organ bath filled with Krebs-Henseleit solution at 37°C and gassed with 95% O₂-5% CO₂.

Denervated urinary bladder preparations (whole or strips) can be used to determine the degree of effect on nervous system inputs. Following appropriate anesthesia, the pelvic plexus is accessed in rats by a ventral midline incision and both pelvic ganglia are obliterated by electrocauterization. The animals are allowed to recover for at least four days before the urinary bladder is harvested as described above; during this period, the neurotransmitters downstream from the nervous system inputs become inactive and only the autonomous inputs remain. Differences in response between the denervated urinary bladder preparation and one harvested from a sham-operated animal are considered to result from neural inputs. The urinary bladders of the denervated animals must be manually emptied on a daily basis during the four day

recovery period (Birder et al. 1998; Brauerman et al. 2006).

Inputs from the urothelium can be assessed in isolated urinary bladder smooth muscle preparations. Following preparation of strips from the urinary bladder, the urothelium is gently peeled away from the cut surface and the strips are mounted as described above. Effects on the capsaicin-mediated nitric oxide pathways located in the urothelium can be assessed in these preparations (Birder et al. 1998). Effects on the release of neurotransmitter substances from the epithelial strips removed from these preparations can also be examined; following isolation, the epithelial strips are incubated in Krebs buffer of suitable balanced electrolyte solution. Following a 20 minute equilibration period, the strips are stimulated either mechanically (by stroking with a glass rod or pinching with forceps) or electrically (using field stimulation of the incubation medium). The neurotransmitter substance of interest is analyzed in the bath solution and dose-response curves are generated (Downie and Karmazyn, 1984).

Ukai et al. (2006) investigated the participation of endogenous endothelin and ET_A receptor in premitigation contractions in rats with bladder outlet obstruction, a model for bladder overactivity.

Weetman (1972) described the preparation of the isolated, innervated urinary bladder in **guinea pigs**. Contractions of the tissue induced by nerve stimulation could be blocked by local anesthetics and by tetrodotoxin.

Isolated innervated, rat and guinea pig hemi-urinary bladder preparations were described by Dhattiwala and Dave (1975).

Burnstock et al. (1978) used recorded isometric tension of mucosal-free strips of the detrusor of the bladder from guinea pigs *in vitro* after electrical field stimulation.

Von Heyden et al. (1997) tested urethral relaxation after electrostimulation in guinea pigs. Male Hartley guinea pigs weighing 350–450 g were sacrificed and bladder, urethra and penis were dissected out. From each animal 4–6 urethral rings 1–2 mm thick were cut. The urethral rings were mounted according to their anatomical order: in channel 1 the most proximal ring (near the bladder neck) and in channel 6 the most distal ring (near the penile crura). The urethral rings were stretched by two spring-wire clips (Harvard Apparatus, South Natick, MA) whose tips closed in the urethral lumen. The manner in which the urethral rings were cut and mounted ensured that only the circularly oriented, mostly striated fibers contributed to the tension measured. Detrusor muscle was cut as a horizon-

tal ring proximal to the trigone and mounted in the same way. The clips were connected with 4-0 silk to a glass tissue support hook on one side and to an isometric force transducer (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) on the other. A double-chambered bath (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) was used in which the working chamber was connected to a second chamber, in which the gas (95% O₂ and 5% CO₂) was fed. The gas flow induced fluid circulation. Forces lower than 0.1 g could be measured without bubble artifacts in the working chamber. The transducer signals were fed into a thermal array recorder (Dash 10, Astro-Med, 63110 Rodgau, Germany). For tissue stimulation, vertical, L-shaped custom-made platinum electrodes (20 mm long, 0.3 mm diameter) 10 mm apart were used with a custom-made stimulator. The tissue was mounted parallel with the electrodes. Bipolar, monophasic balance-charged rectangular pulses of 0.8-ms duration and 75 mA current were used.

Kunisawa et al. (1985) performed a pharmacological study of alpha adrenergic receptor subtypes in smooth muscle of **human** urinary bladder base and prostatic urethra.

Wuest et al. (2005) studied the effect of a potassium channel opener on contraction of detrusor strips from **mouse, pig** and **man** in the presence and absence of urothelium..

Thornbury et al. (1992) reported on the mediation of nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in **sheep**. Urinary bladders of sheep of either sex were obtained approximately 15 min after slaughter. Circularly oriented rings were cut from the region of the bladder just above the trigone. These were opened and the mucosa removed by sharp dissection to give strips with approximate dimensions of 10 × 4 × 4 mm. The strips were mounted in organ baths and perfused with Krebs solution. Tension was measured with isometric transducers after field stimulation via platinum ring electrodes.

Hills et al. (1984) used isolated strips of the bladder from female **pigs**. Bladder neck strips were cut longitudinally and horizontally from the region of the bladder just below the trigone. The preparations were stripped of mucosa and trimmed to give a muscle strip of about 2 × 15 mm.

Klarskov (1987) studied the non-cholinergic, non-adrenergic inhibitory nerve responses of bladder outlet smooth muscle from female Danish Landrace pigs *in vitro*. Trigone strips were taken in an oblique direction from the internal urethral orifice and medially to one of the ureteric orifices, bladder neck strips transversal

from the posterior half of the borderline between bladder and urethra, and urethral strips longitudinal from the proximal posterior part.

Teramoto et al. (1997) examined the membrane potential in the proximal urethra of pigs by use of the microelectrode technique.

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C.5.2.4

Effects on Isolated Urethra

PURPOSE AND RATIONALE

The relaxation response in the urethra results in a combination of decreased intraurethral pressure, increased urethral diameter and urethral shortening resulting in a flaring of the urethral orifice. A number of neurotransmitters and mediators are involved in this process, with each affecting a different stage of the process.

(Brading 1999; Andersson and Wein, 2004) The urethra is difficult to study *in situ* because of the large amount of surrounding connective tissue, which can be overcome by the use of a whole-mount preparation (Jankowski et al. 2004).

PROCEDURE

Female rats or guinea pigs have been used. Following appropriate anesthesia, the bladder and urethra are exposed via a lower midline incision, and a catheter is inserted in the urethral lumen, extending the entire axial length and exiting from a hole placed in the bladder dome. The urethra is secured to the tubing with sutures at both ends to maintain the correct *in vivo* length after dissection, and is measured. The pubic bone is then cut at a position lateral to the urethra and then separated and resected. The exposed urethra was gently removed from the ventral vaginal wall and the whole bladder-urethra unit was immediately placed in cold, oxygenated suitable balanced electrolyte solution bubbled with 21% O₂-5% CO₂-74% N₂.

The catheter is removed so that the urethra may be secured inside the experimental apparatus, which provides a controlled fixed intraluminal pressure via an adjustable static fluid reservoir (the intraluminal pressure is controlled by adjusting the height of the reservoir). The mounted urethra is then enclosed in a bathing chamber filled with the same gassed, balanced electrolyte solution at 37°C. The preparation is allowed to equilibrate for at least 30 min before testing. A laser micrometer is positioned to measure urethral outer diameter at chosen locations along the axial length. Proximal, mid, and distal regional measurements were performed by positioning the laser at axial positions 25, 50, and 75%, respectively, from the apex of the bladder, based on *in vivo* length. Both pressure and outer diameter (OD) measurements are recorded simultaneously.

The test compound of interest is added to the bath and pressure and OD measurements are obtained following a 30 minute equilibration period. Pressure-diameter (P-D) responses are generated by incrementally increasing the pressure, in 2 mm Hg steps, from 0 to 18 mm Hg, and OD data are collected at 10 Hz over a 1-min period for each 2-mmHg step. The OD data are then averaged for each incremental increase to obtain a discrete value for each value of applied pressure.

Contractile responses can be evaluated in this preparation as well. The urethra is exposed to a fixed intraluminal pressure of 8 mmHg, which causes the tissue to be predilated and allows for a contractile response to be generated. The OD resulting from this 8-mmHg

applied pressure is measured at a single axial location (i. e., proximal, mid, or distal) 30 min after pressurization. The test chemical of interest is added to the bath and the preparation is exposed for 30 minutes; at the end of this time, a series of 100 OD measurements (taken at 1 Hz) are collected at the same location and are averaged.

EVALUATION

The P-D response data are used to generate tissue compliance (C) as follows:

$$C = [D_{\max} - D_{\min}/D_{\min}] \times (P_{\max} - P_{\min})^{-1}$$

where D_{\max} and D_{\min} represent the OD at the maximum (P_{\max} , 18 mm Hg) and minimum (P_{\min} , 0 mm Hg) applied pressures, respectively.

Contractile responses are assessed by determining the percent change in mean OD before and after the addition of the test compound.

MODIFICATION OF THE METHOD

With careful dissection, the pudendal nerves can be identified and left intact during the dissection of the urethra from the guinea pig. These nerves can then be attached to electrodes and alterations of effects of neural inputs can be assessed. In this preparation, contractile responses were assessed by measuring changes in isometric tension and intraluminal pressure changes were also recorded (Walters et al., 2006).

Triguero et al. (2003) studied the relaxation effects of scorpion venom in the isolated urethra of **sheep**.

Michel et al. (2006) performed *in vivo* studies on the effects of α_1 -adrenoceptor antagonists on pupil diameter and urethral tone in **rabbits**.

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C.5.2.5

Effects on External Urethral Sphincter

PURPOSE AND RATIONALE

In contrast to the smooth musculature of the internal urethral sphincter, the external urethral sphincter is a striate muscle and a part of the urogenital diaphragm (Kuru 1965). Judged from electromyographic studies it is generally accepted that the preponderance of continence depends on the external urethral sphincter. The external urethral sphincter shows a steady tonic discharge under resting conditions. As the bladder fills, there is initially an increase in this activity. When the rise in tension in the bladder wall leads to reflex contraction, the activity in the external urethra sphincter ceases and it remains quiescent during voiding. Parlani et al. (1992) used the external urethral sphincter of the rat as an *in vitro* model to evaluate the activity of drugs on the smooth and striated components of the urinary bladder outlet.

PROCEDURE

Male Wistar rats weighing 360–400 g are sacrificed by decapitation and exsanguination. Through a midline incision of the lower abdomen, the external urethral sphincter (Watanabe and Yamamoto 1979) is isolated from the perineal muscles and surrounding connective tissue and removed *in toto*. The preparation is placed in oxygenated Krebs solution, and a ring is taken from its middle region. In this area the urethra is encircled by bundles of striated muscle fibers partly interlaced with urethral smooth muscle. The rings are cut to obtain strips that are suspended in a 5-ml organ bath containing Krebs solution at 37°C. A mixture of 96% O₂ and 4% CO₂ is bubbled into the organ bath. The preparations are connected by means of a silk thread to an isometric strain gauge under a constant load of 1 g. The contractile activity is recorded on a polygraph. Field stimulation is carried out by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a Grass S11 stimulator. The preparations are allowed to equilibrate for at least 60 min. Square wave pulses are delivered at an intensity between 10 and 60 V, a frequency between 0.1 and 3 Hz, and a duration between 0.1 and 1 s; trains are of 5 s every 5 min. After three consecutive reproducible responses are obtained, drugs are added to the organ bath. The effect of drugs is expressed as the percent of

inhibition of contractile response before exposure to drugs and is evaluated as soon as the maximum effect is reached.

EVALUATION

Means ± standard error of the mean are calculated. Statistical evaluation is performed by using Student's *t*-test for paired or unpaired data.

MODIFICATIONS OF THE METHOD

In some studies, denervation of the external urethral sphincter was performed (Somma et al. 1989; Parlani et al. 1992). Rats were anesthetized with 30 mg/kg penthotal *i.p.*, then the major pelvic ganglia, known to provide both sympathetic and parasympathetic innervation to the urinary bladder and the external urethral sphincter (Hulsebosh and Goggeshall 1982; Purinton et al. 1973; Watanabe and Yamamoto 1979) are isolated and bilaterally removed through a small incision of the lower abdomen.

In the same preparations, somatic denervation of the external urethral sphincter was obtained by cutting the pudendal nerves. The paravertebral muscles were carefully dissected through an incision of the skin to exteriorize the sacral plexus. The pudendal nerves were isolated, and 2–3 mm of the nerve were removed. An absorbable sponge soaked with amikain solution was left in place to prevent bleeding and infection. The muscles and the skin were sutured with cat gut. The rats were allowed to recover for 10 to 15 days in individual cages with free access to water and food.

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Chapter D

Respiratory Activity

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| D.2.2.7 | Isolated Larynx In Situ | 533 | | Histamine receptors have been classified on the ba- sis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H ₁ receptor couples mainly to G _{q/11} , thereby stimulating phospholipase C, whereas the H ₂ receptor interacts with G _s to activate adenylyl cy- clase. The histamine H ₃ and H ₄ receptors couple to G _i proteins to inhibit adenylyl cyclase, and to stimulate MAPK (Hough 2001). | | | |
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say is used to determine the affinity of test compounds to the histamine H₁ receptor by measuring their inhibitory activities on the binding of the H₁ antagonist ³H-pyrilamine to a plasma membrane preparation from guinea pig brain.

PROCEDURE

Brains from guinea-pigs are homogenized in ice-cold Tris buffer (pH 7.5) in a Potter homogenizer (1 g brain in 30 ml buffer). The homogenate is centrifuged at 4°C for 10 min at 50,000 g. The supernatant is discarded, the pellet resuspended in buffer, centrifuged as before, and the final pellets resuspended in Tris buffer (1 g fresh weight/5 ml). Aliquots of 1 ml are frozen at -70°C.

In the competition experiment, 50 μl ³H-pyrilamine (one constant concentration of 2 × 10⁻⁹ M), 50 μl test compound (>10 concentrations, 10⁻⁵–10⁻¹⁰ M) and 100 μl membrane suspension from guinea pig whole brain (approx. 10 mg wet weight/ml) per sample are incubated in a shaking bath at 25°C for 30 min. Incubation buffer: 50 mM Tris-HCl buffer, pH 7.5.

Saturation experiments are performed with 11 concentrations of ³H-pyrilamine (0.1–50 × 10⁻⁹ M). Total binding is determined in the presence of incubation buffer, non-specific binding is determined in the presence of mepyramine or doxepin (10⁻⁵ M).

The reaction is stopped by rapid vacuum filtration through glass fibre filters. Thereby the membrane-bound is separated from the free radioactivity. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION OF RESULTS

The following parameters are calculated:

- total binding of ³H-pyrilamine
- non-specific binding: binding of ³H-pyrilamine in the presence of mepyramine or doxepin
- specific binding = total binding – non-specific binding
- % inhibition of ³H-pyrilamine binding: 100 – specific binding as percentage of control value

The dissociation constant (*K*₁) and the *IC*₅₀ value of the test drug are determined from the competition experiment of ³H-pyrilamine versus non-labeled drug by a computer-supported analysis of the binding data.

MODIFICATIONS OF THE METHOD

De Backer et al. (1993) reported genomic cloning, heterologous expression in COS-7 cells and pharmacological characterization of a human H₁-receptor.

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D.1.0.2

Muscarinic Receptor Binding

PURPOSE AND RATIONALE

Muscarinic receptors in the airways are important in both the normal physiology and the pathophysiology of pulmonary function. Acetylcholine released from parasympathetic nerve terminals causes contraction of airway smooth muscle. Animals with asthma or other chronic inflammation of the airways exhibit hypersensitivity of the airways to muscarinic agonists, and muscarinic antagonists are used therapeutically in patients with asthma and chronic obstructive pulmonary disease (Nathanson 2000). Muscarinic receptors are present in neurons in the central and peripheral nervous system, cardiac and smooth muscles, and a variety of exocrine glands. Mammals possess genes encoding five different subtypes of mAChR, termed M₁–M₅, which can be divided into two broad functional categories: the M₁, M₃, and M₅ receptors preferentially couple to the G_q family of G-proteins whereas the M₂ and M₄ receptors preferentially couple to the G_i family of G-proteins.

For involvement of acetylcholine receptors in the gastrointestinal tract see J.3.6.1.2.

Several papers deal with the distribution of muscarinic receptors in the lung, their role in pulmonary disease and the use of muscarinic antagonists for the

treatment of obstructive airways disease (Mak and Barnes 1990; Barnes 1993, 2001, 2004; Disse 2001; Disse et al. 1993, 1999; Haddad et al. 1994; Barnes et al. 1995, 1997; Patel et al. 1995; Peták et al. 1996; Alabaster 1997; Matsumoto 1997; Chelala et al. 1998; Hislop et al. 1998; Okazawa et al. 1998; Wale et al. 1999; Rees 2002; Sarria et al. 2002; Tohda et al. 2002; Costello et al. 2006).

Hirose et al. (2001) described the pharmacological properties of a muscarinic antagonist with M₂-sparing antagonistic activity.

PROCEDURE

Binding Affinity for Human and Rat Muscarinic Receptor Subtypes

In competition studies, specific binding of [³H]N-methylscopolamine (NMS; New England Nuclear, Boston, Mass., USA) was determined using membranes from Chinese hamster ovary (CHO) cells expressing cloned human m1, m2, m3, m4, or m5 receptors (Receptor Biology, Baltimore, Md., USA), rat m1 or m3 receptors (American Type Culture Collection, Manassas, Va., USA), and rat heart tissue. These CHO cells expressing cloned rat m1 or m3 receptors, and rat heart tissue were homogenized in 3 vols of 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA containing 20% sucrose with a Polytron PT-10. The homogenates were centrifuged at 10,000 g for 30 min at 4°C. The supernatants were centrifuged at 100,000 g for 60 min at 4°C. The pellets were suspended in 50 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂ and centrifuged at 100,000 g for 60 min at 4°C. The pellets were re-suspended in the above-mentioned buffer (25 mg/ml for CHO cells expressing cloned rat m1 or m3, and 50 mg/ml for rat heart tissue) and stored at -80°C as membrane preparations. In the binding assay, the membrane preparations were incubated with 0.19 to 0.2 nM [³H]NMS in 50 mM Tris-HCl, 10 mM MgCl₂, and 1 mM EDTA (pH 7.4) for 2 h at room temperature. Final protein concentrations were 22 µg/ml (human m1), 70 µg/ml (human m2), 54 µg/ml (human m3), 20 µg/ml (human m4), 116 µg/ml (human m5), 481 µg/ml (rat m1 and m3), and 2500 µg/ml (rat heart). Assays were performed in a total volume of 500 µl. Non-specific binding was measured in the presence of 1 µM NMS and it was less than 2% of total binding. Free and membrane-bound [³H]NMS were separated by filtration over glass filters (UniFilter-GF/C; Packard Instruments, Meriden, Conn., USA) using a cell harvester (Filtermate 196; Packard Instruments). Radioactivity was counted by a liquid scintillation counter (TopCount; Packard Instruments).

EVALUATION

For saturation studies, membranes from CHO cells expressing human m3 were incubated with an increased concentration of [³H]NMS (0.1–3.2 nM) in the presence or absence of 10 nM compound A, and specific binding of [³H]NMS was determined after incubation for 2 h.

Competition binding data were analyzed by a non-linear regression fitting program using GraphPad Prism Software (San Diego, Calif., USA). Saturation binding data were transformed to make a Scatchard plot and analyzed by a linear regression fitting program using GraphPad Prism Software.

The K_i values were calculated from the IC₅₀ values by using the following equation:

$$K_i = IC_{50} / (1 + [L] / K_d)$$

where K_d is the dissociation constant of [³H]NMS in each receptor subtype, and [L] is the concentration of [³H]NMS (Cheng and Prussoff 1973). K_d values of [³H]NMS in each receptor subtype were determined by Scatchard plot analysis. The K_d and B_{max} values below were used in this study. Data of human cloned receptors were extracted from Receptor Biology's Product Information Sheets (Receptor Biology).

| | | |
|-------------------|-------------------------|-------------------------------------------------|
| Human m1 receptor | K _d = 51 pM | B _{max} = 1.28 pmol/mg of protein |
| Human m2 receptor | K _d = 290 pM | B _{max} = 1 pmol/mg of protein |
| Human m3 receptor | K _d = 86 pM | B _{max} = 0.65 pmol/mg of protein |
| Human m4 receptor | K _d = 56 pM | B _{max} = 1.44 pmol/mg of protein |
| Human m5 receptor | K _d = 200 pM | B _{max} = 0.59 pmol/mg of protein |
| Rat m1 receptor | K _d = 62 pM | B _{max} = 0.039 pmol/mg of wet weight |
| Rat m2 receptor | K _d = 210 pM | B _{max} = 0.0090 pmol/mg of wet weight |
| Rat m3 receptor | K _d = 72 pM | B _{max} = 0.019 pmol/mg of wet weight |

In saturation studies, the K_i value was calculated using the following equation:

$$K_i = K_d / (K'_d - K_d) \times [C]$$

where K'_d or K_d is the dissociation constant of [³H]NMS in human m3 receptors in the presence or absence of an inhibitor, respectively, and [C] is the concentration of the test drug (Nishikibe et al. 1999).

MODIFICATIONS OF THE METHOD

Struckmann et al. (2003) investigated the role of muscarinic receptor subtypes in the constriction of peripheral airways by studies on receptor-deficient mice.

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D.2**Effects on Air Ways****D.2.1****Tests in Isolated Organs****D.2.1.1****Spasmolytic Activity in Isolated Guinea Pig Lung Strips****PURPOSE AND RATIONALE**

Several autacoids such as histamine and leukotrienes, induce bronchoconstriction. Histamine is an important mediator of immediate allergic and inflammatory reactions. It causes bronchoconstriction by activating H₁-receptors. Calcium ionophores induce the release of leukotrienes via the 5-lipoxygenase pathway. Leukotrienes are powerful bronchoconstrictors that appear to act on smooth muscles via specific receptors. In this method, drugs are tested for their capability of inhibiting bronchospasm induced by histamine or calcium ionophore. It is used to detect H₁- and leukotriene receptor blocking properties of test compounds.

PROCEDURE

Albino guinea-pigs of either sex weighing 300–450 g are sacrificed with an overdose of ether. The chest cavity is opened and the lungs are removed. They are cut into strips of 5 cm and placed into a physiological saline solution. Thereafter, the lung strips are mounted in an organ bath containing a nutritive solution. The bath is bubbled with carbogen and maintained at 37°C. Under a pre-load of 0.5 g–3 g, the tissue is left to equilibrate for 30–60 min. Prior to testing, carbachol is added to the bath to test the lung strips' ability of contraction. Twenty min later, two prevalues are obtained by adding the spasmogen

- histamine dihydrochloride 10^{-6} g/ml for 5 min, or
- Ca²⁺ ionophore 5×10^{-6} g/ml for 5 min, or
- Leukotriene LTC₄ 10^{-9} – 10^{-8} g/ml for 10 min, or
- Leukotriene LTD₄ 10^{-9} – 10^{-8} g/ml for 10 min

to the bath and recording the contractile force at its maximal level. Following a 20 min equilibration period, the spasmogen is administered again. Five minutes thereafter, the test compound is added in cumulative doses from 10^{-8} to 10^{-4} g/ml at 5 or 10 min intervals. The contractile response is determined isometrically.

Test Modification: Inhibition of Prostaglandin Synthesis

This procedure is identical to the test described above with the exception that the prostaglandin synthesis is inhibited by addition of indomethacin at 10^{-6} g/ml prior to spasmogen administration.

EVALUATION

The percent inhibition of spasmogen induced contraction is calculated.

MODIFICATIONS OF THE METHOD

Lung parenchyma strips from various species were used to measure bronchoactivity by Kleinstiver and Eyre (1979).

A descriptive model of the events occurring during an inflation-deflation cycle using excised rat lungs was proposed by Frazer et al. (1985).

Barrow (1986) measured volume-pressure cycles in air-filled or liquid-filled rabbit lungs ranging from intact lungs with the rib cage immobilized to isolated lungs.

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D.2.1.2

Spasmolytic Activity in Isolated Trachea

PURPOSE AND RATIONALE

The isolated tracheal chain of guinea pigs can be used to test for β -blocking activity (see Sect. A.1.2.7). In addition, this model can be used to test compounds which inhibit bronchospasms. It is used to detect β -sympathomimetic, H₁-receptor blocking and leukotriene receptor blocking properties of test drugs.

Carbachol is a cholinergic agonist that produces contraction of bronchial smooth muscle by muscarinic stimulation.

Histamine is an important mediator of immediate allergic (type 1) and inflammatory reactions. It causes bronchoconstriction by activating H₁-receptors.

Calcium ionophores induce the release of leukotrienes via the 5-lipoxygenase pathway. Leukotrienes are powerful bronchoconstrictors that appear to act on smooth muscle via specific receptors.

To assess a compound's ability to inhibit carbachol induced bronchospasm via β -receptor activation, a β -receptor blocking agent (for example propranolol) must be added. If relaxation of bronchial smooth muscle is brought about by β -receptor activation, the spasmolytic effect will decrease following propranolol administration.

The effect of bradykinin can be abolished by bradykinin antagonists (Hock et al. 1991).

The effects of potassium channel openers can also be studied in this test (Englert et al. 1992).

PROCEDURE

Albino guinea pigs of either sex weighing 300–550 g are sacrificed by CO₂ narcosis. The entire trachea is

dissected out and cut into individual rings (2–3 cartilaginous rings wide). Twelve–fifteen rings are tied together with silk threads and mounted in the organ bath containing Krebs–Henseleit solution. The tissue is maintained at 37°C under a tension of 0.5 g and gassed with carbogen. Isometric contractions are recorded via a strain-gauge transduced on a polygraph. Forty-five minutes are allowed for equilibration before the addition of the spasmogen.

The following spasmogens are used:

- carbachol (2×10^{-7} g/ml)
- histamine (10^{-7} g/ml)
- Ca – ionophore for release of leukotrienes
- leukotriene LTC₄ (10^{-9} – 10^{-8} g/ml)
- leukotriene LTD₄ (10^{-9} – 10^{-8} g/ml)

When the contraction has reached its maximum (initial spasm) after 10–12 min, the standard drug, e. g., isoprenaline (1 ng/ml) or aminophylline (10 ng/ml) is administered. The bronchial responses are allowed to plateau and are recorded. The tissue is rinsed thoroughly and control contractions are induced again by adding spasmogen. After obtaining the initial spasm again, the test drug is added and the contractile force is recorded at its maximal level.

Determination of mechanism of action (testing for β -sympathomimetic effect). After obtaining the initial carbachol induced spasm, propranolol is administered 5 min before the addition of the test drug. Three minutes later, the tissue is challenged by carbachol administration.

EVALUATION

The percent inhibition of carbachol or other spasmogen induced contractions is calculated. From dose-response curves ED_{50} values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The isolated guinea pig trachea has been proven to be an useful tool for several purposes, e. g., screening procedures and studies on mode of action, e. g., of potassium channel openers.

MODIFICATIONS OF THE METHOD

The molecular mechanisms of β -adrenergic relaxation of airway smooth muscle were described by Kotlikoff and Kamm (1996).

Farmer et al. (1986) studied the effects of epithelium removal on the sensitivity of **guinea-pig** isolated trachealis to bronchodilator drugs.

Wilkins et al. (1992) described a bioassay system for a tracheal smooth muscle-constricting factor using

an isolated guinea pig trachea which was cannulated from both sides, mounted in an organ bath and monitored by a TV camera attached to a microscope. The picture was digitized continuously, and the diameter of the trachea calculated and displayed on a monitor throughout the experiment.

Coleman and Nials (1989) described a versatile, eight-chamber superfusion system for the evaluation of spasmogenic and spasmolytic agents using guinea pig isolated tracheal smooth muscle.

Goldie et al. (1986a) studied the influence of the epithelium on responsiveness of guinea pig isolated trachea to contractile and relaxant agonists.

Lee et al. (1997) studied the effects of bupivacaine and its isomers on guinea pig tracheal smooth muscle. The trachea from the larynx to the carina was removed and cut into single rings. Seven tracheal rings were tied together with the circular muscle running on the same side of the chain, placed into an organ bath containing Krebs Ringer's solution and connected to a force placement transducer for measurement of isometric tension.

Wong et al. (1997) tested the effects of tyrosine kinase inhibitors on antigen challenge of guinea pig lung *in vitro*. Guinea pigs were passively sensitized by a single i.p. injection of 1 mg/kg rabbit IgG antibody against ovalbumin. Bronchial rings, 3 mm in length, were obtained from the hilar bronchi of sacrificed animals and suspended isometrically in an organ bath in Krebs bicarbonate buffer with a resting load of 2 g. To determine maximum antigen-induced contractions, bronchial rings were exposed to increasing concentrations of ovalbumin. To evaluate the role of protein tyrosine kinase in mediating smooth muscle anaphylactic contraction, protein tyrosine kinase inhibitors were preincubated with bronchial rings 30 min before addition of ovalbumin.

Eltze and Galvan (1994) compared the inhibition of preganglionic and postganglionic contraction of the **rabbit** isolated bronchus/trachea by antagonists with selectivity for different muscarinic receptor subtypes with their affinities at M₁, M₂, M₃, and M₄ receptors (Barnes 1993).

For **experiments with vagus nerve stimulation**, the vagi were isolated with rings of the proximal main stem bronchi and a small portion of the distal trachea. The tissues were hung on stainless steel hooks, which passed through the lumen, and were placed in a water-jacketed organ bath filled with Krebs solution plus 2×10^{-5} M choline chloride to promote resynthesis of acetylcholine, 10^{-5} M indomethacin to prevent generation of cyclo-oxygenase products, and 10^{-6} M

DL-propranolol to block possible β -adrenoceptor-mediated effects. The preparation was fixed under a resting tension of 1 g for isometric contraction measurement using a force transducer. The vagi were passed around bipolar platinum electrodes held at the surface of the bath. Electrical stimulation was performed with trains (20 Hz, 0.3 ms at 20 V) elicited every 20 min.

For experiments with **field stimulation**, two-ring preparations from the distal trachea of the rabbit were suspended in organ bath in Krebs solution with the above mentioned additions under a resting tension of 1 g. Isometric contractions were elicited at 1 h intervals by continuous electrical field stimulation via platinum electrodes (20 Hz, 0.3 ms at 20 V) for an average 3–7 min to reach a stable plateau.

Vaali et al. (1996) studied in isolated tracheal rings of **rats** and guinea pigs the bronchorelaxing effects of nitric oxide donors. The epithelium of some rings was removed by gentle rubbing. Rat mesenteric rings were cut from the same animals as the bronchi and similarly prepared by removing the endothelium by gently rubbing of the intimate surface.

Farmer et al. (1994) used the isolated trachea from male **ferrets** weighing 1.5–2.5 kg to study the effects of bradykinin receptor agonists.

Toews et al. (1997) assessed the effects of the phospholipid mediator lysophosphatidic acid on the contractile responsiveness of isolated tracheal rings from **rabbits** and **cats**.

Tamaoki et al. (1993) used isolated rings of segmental bronchi from **dogs** to study atypical β -adrenoceptor- (β_3 -adrenoceptor) mediated relaxation.

The preparation of **bovine** tracheal smooth muscle for measuring airway responsiveness *in vitro* was described by Hashjin et al. (1995).

Wali (1987) used tracheal strips from 2- to 4-week-old **chicks** to study inhibition of cholinergic and non-cholinergic neural and muscular contractions by local anesthetics.

Lulich and Paterson (1980) used **human** isolated bronchial muscle preparations and compared the effects of histamine and other drugs with the effects observed on the central and peripheral airways of the rat.

Goldie et al. (1986b) measured the responses of human bronchial strip preparations to contractile and relaxant agonists in preparations from non-diseased and from asthmatic lung obtained 3–15 h post mortem.

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D.2.1.3

Reactivity of the Isolated Perfused Trachea

PURPOSE AND RATIONALE

The mechanism by which the epithelium affects the reactivity of tracheal musculature can be studied using the isolated perfused trachea preparation. Contractile agonists can be added either to the serosal (extraluminal) or to the mucosal (intraluminal) surface (Fedan and Frazer 1992).

PROCEDURE

A 4-cm segment of the trachea of male guinea pigs is removed after sacrifice of the animal and placed for cleaning in modified Krebs-Henseleit solution at 37°C containing (millimolar) NaCl 113.0, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, and glucose 5.7 (pH 7.4) being gassed with 95% O₂/5% CO₂. The trachea is then attached to a stainless-steel perfusion holder (Munakata et al. 1988), extended to its *in situ* length and placed in an organ chamber (the serosal compartment) at 37°C containing 25 ml of gassed modified Krebs-Henseleit solution. This solution is also pumped at a constant rate of 30 ml/min through the lumen (mucosal compartment). Responses of the tracheal musculature are obtained by measuring changes in inlet-outlet ΔP between the side holes of indwelling catheters, while the trachea as per-

fused at a constant rate of modified Krebs-Henseleit solution. The inlet and outlet catheters are connected to the positive and negative sides, respectively, of a differential pressure transducer.

Agonists are added in step-wise increasing, cumulative concentrations. Two consecutive dose-response curves are obtained after the addition either to the serosal or mucosal compartment. The second dose-response curve is obtained 1.5 h after the end of the first, the preparation being washed every 15 min during the intervening period.

EVALUATION

Responses are quantified as ΔP in centimeters of H₂O. Geometric EC₅₀ values are determined from least-square analysis of a logit model and are presented along with 95% confidence intervals.

MODIFICATIONS OF THE METHOD

Baersch and Frölich (1996) measured continuously the changes of the diameter of isolated guinea pig tracheal tubes by a newly developed imaging bioassay system. The tracheal tube between larynx and bifurcation was prepared to a length of 1.8–2.5 cm, cannulated at both ends and mounted in a 15 ml organ bath filled and perfused with oxygenated Krebs solution at 37°C. The lumen of the trachea was perfused with warm (37°C) Krebs solution from a reservoir that was isolated from the tissue bath. Changes in diameter of the trachea were assessed by computerized video microscopy. The tracheal diameter was used as marker for airway size, thus allowing calculation of muscle contractions under experimental conditions. Following a 30-min equilibrium period, a contraction was induced by intraluminal application of methacholine (0.1 mmol/l) as reference contraction. After washout and return to a stable baseline, cumulative concentration-response curves were obtained by luminal or extraluminal drug application.

Yang et al. (1991) studied the role of epithelium in airway smooth muscle responses to relaxant agents. The results suggested that the epithelium is a relatively weak barrier for lipophilic agents but has a major role as a diffusion barrier to hydrophilic substances.

Munakata et al. (1988, 1989) developed an *in vitro* system to assess the role of epithelium in regulating airway tone using the intact **guinea pig** trachea. The responses to histamine, acetylcholine and hypertonic KCl when stimulated from the epithelial or serosal site were first examined in tracheae with intact epithelium. Then the responses to these agonists were registered after epithelial denudation.

Pavlovic et al. (1989) studied the role of airway epithelium in the modulation of bronchomotor tone in the isolated trachea of **rats**. An organ bath was constructed that permitted independent circulation of fluid within the lumen or around the exterior of the tracheal segment. In one-half of the preparations the epithelium was mechanically removed.

Fernandes et al. (1989) described a co-axial bioassay system, whereby rat cross-cut aorta strip preparations were set up in co-axial assemblies under 500 mg resting tension within a guinea pig tracheal segment serving as donor of the smooth muscle relaxant factor from guinea-pig tracheal epithelium.

Lewis and Broadley (1995) investigated the influence of spasmogen inhalation by guinea pigs upon subsequent demonstration of ovalbumin-induced hyperreactivity in isolated airway tissues. Guinea pigs were sensitized with ovalbumin (i.p.) 14 days before use. *In vitro* airway hyperreactivity induced by ovalbumin inhalation was determined by challenging with aerosolized spasmogen (5-HT, methacholine, the thromboxane-mimetic U-46619, or adenosine) 24 h before and again 18–24 h after the ovalbumin inhalation. One h later, the animals were sacrificed and isolated airways perfused lung halves and tracheal spirals were set up for determination of tissue sensitivity to carbachol, histamine, and adenosine.

Sparrow and Mitchell (1991) used bronchial segments obtained from the lungs of Large-white-/Land-race-cross **pigs** to study the modulation by the epithelium of the extent of bronchial narrowing produced by substances perfused through the lumen.

Mitchell et al. (1989) compared the reactions of perfused bronchial segments and bronchial strips of **pigs** to histamine and carbachol.

Hulsman et al. (1992) recommended the perfused **human** bronchiolar tube as a suitable model.

Omari et al. (1993) studied the responsiveness of human isolated bronchial segments and its relationship to epithelial loss.

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D.2.1.4

Bronchial Perfusion of Isolated Lung

PURPOSE AND RATIONALE

Bronchial perfusion of the isolated lung was described by Sollmann and von Oettingen (1928) as a simple method for studying pharmacological reactions of bronchiolar muscle. The method consists in perfusing fluid down the trachea through the bronchi, and allowing it to escape from the alveoli through scratches on the surface of the lungs. Bronchoconstriction results in a reduced rate of flow, bronchodilatation is indicated by an increased flow. The method has been used to evaluate sympathomimetic drugs by Tainter et al. (1934) and by Luduena et al. (1957).

PROCEDURE

Guinea pigs weighing about 200 g are sacrificed by a head blow. The chest is opened, the trachea cut at the upper end and removed with the lung. The trachea is attached to the cannula of an perfusion apparatus. Only one lung is perfused, the other being tied off. The lower part of the lower lobe is cut off and the rest of the lung surface is scratched deeply assuring maximal pre-medication flow.

The perfusion fluid has the following composition in percentage of anhydrous salts: NaCl 0.659, NaHCO₃ 0.252, KCl 0.046, CaCl₂ 0.005, MgCl₂ 0.0135, NaH₂PO₄ 0.01, Na₂HPO₄ 0.008, glucose 5%, pH 8.0. The temperature of the perfusion medium is 37.5°C and the lung is enclosed in

a glass cylinder to be protected from variations in the environmental temperature.

The trachea is attached to the cannula of a perfusion apparatus which pumps the solution at a constant rate into a manometric tube connected with the perfused organ. Resistance to the flow (bronchoconstriction) results in an increase in the height of the column of fluid in the manometer. The intensity of bronchodilator effect is measured by the fall of the column in the manometer.

After the lung is attached to a T-shaped cannula, the pump is set in motion and the fluid, after filling the lung, flows out of the system through the third opening of the cannula. By gentle pressure air bubbles are forced out of the lung into the overflow. The lung is then treated in the aforesaid manner, and the upper outlet of the cannula closed. Histamine HCl is added in a concentration of 1:2500000 as soon as the perfusion starts and the flow is adjusted to obtain a constant progressive increase in pressure.

The drugs are injected near the cannula when the perfusion pressure reaches a level of 500–650 ml of water. The volume injected is always 0.1 ml.

Each drug is tested for bronchodilating activity against the bronchoconstriction induced by histamine in parallel with l-arterenol following a Latin square, including three doses of each drug and three doses of l-arterenol graded at 0.5 log intervals.

EVALUATION

Activity ratios of bronchodilating agents versus the standard can be calculated with a 3 + 3 point assay including confidence limits.

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D.2.1.5

Vascular and Airway Responses in the Isolated Lung

PURPOSE AND RATIONALE

The isolated perfused rat lung allows the simultaneous registration of pulmonary vascular and airway responses to various drugs.

PROCEDURE

Male Sprague-Dawley rats weighing 300–350 g are intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg). The trachea is cannulated with a short section of polyethylene tubing, connected to a rodent ventilator, and ventilated with room air enriched with 95% O₂/5% CO₂, with a tidal volume of 4–5 ml/kg and 2 cm H₂O positive end-respiratory pressure. The rats are heparinized with 1000 units of intravenous heparin and are rapidly exsanguinated by withdrawing blood from the carotid artery.

The lung is exposed by median sternotomy, and a ligature is placed around the aorta to prevent systemic loss of blood. The main pulmonary artery is catheterized, and the lung is removed en block and suspended in a warmed (39°C), humidified (100%) water-jacketed chamber. An external heat exchanger is used to maintain the temperature of the perfusate and the isolated lung chamber constant throughout the experiment. The perfusate solution (15 ml of heparinized blood and 5 ml modified Krebs-Henseleit solution) is placed in a reservoir and mixed constantly by a magnetic stirrer. The lungs are perfused with a peristaltic roller pump at a flow rate of 8–14 ml/min to maintain a physiological baseline pulmonary arterial perfusion pressure of 15 ± 0.5 mmHg. Pulmonary arterial perfusion pressure, airway pressure, and reservoir blood level are continuously monitored, electronically averaged and recorded with a polygraph.

EVALUATION

Changes (increase or decrease) in pulmonary arterial pressure and in airway pressure after injection of test compounds are measured in mmHg and compared with baseline values.

MODIFICATIONS OF THE METHOD

Bernard et al. (1997) described an isolated perfused lung model with real time data collection and analysis of lung function. Male Sprague Dawley rats were anesthetized with 130 mg/kg pentobarbital i.p. The trachea was cannulated and then ventilated with 5% CO₂ and 95% air at a rate of 60 breath/min and a tidal volume of 2.5 ml. An injection of 650–700 units/kg of heparin was made into the right ventricle. A cannula was placed into the main pulmonary artery. The left ventricle was incised and the lungs were washed free of blood with warmed Krebs-Henseleit bicarbonate buffer with 4.5% BSA and 0.1% glucose. The left atrium was then cannulated to allow outflow of the perfusate. The lung was then removed and suspended in a chamber for perfusion. The flow rate of the perfusate

was adjusted to 8–10 ml/min/kg. Ventilation was maintained at 60 breaths/min with humidified and warmed gas. The lung was allowed to recover for 15 min at which time the lung mechanic parameters of flow, volume, transpulmonary pressure, pulmonary artery pressure, weight, resistance, elastance, and positive end-tidal pressure were measured.

Hauge (1968) studied the conditions governing the pressor responses to ventilation hypoxia in isolated perfused rat lungs.

Uhlig and Heiny (1995) measured the weight of the isolated perfused rat lung during negative pressure ventilation for quantitating edema formation in the isolated lung.

Uhlig and Wollin (1994) described an improved setup for the isolated perfused rat lung. Breathing mechanic, such as tidal volume, pulmonary compliance, and pulmonary resistance, as well as perfusate characteristics, such as pulmonary vascular resistance, pulmonary pre- and postcapillary resistance, perfusate pH, P_{O_2} , and P_{CO_2} , and the capillary filtration coefficient were determined.

Byron et al. (1986) used the isolated perfused rat lung preparation for the study of aerosolized drug deposition and absorption.

Hendriks et al. (1999) published a modified technique of isolated left lung perfusion in the rat.

Riley et al. (1981) determined the tissue elastic properties of saline-filled isolated hamster lungs by measuring the pressure-volume relationships and studied the prevention of bleomycin-induced pulmonary fibrosis by *cis*-4-hydroxy-L-proline.

Lewis and Broadley (1995) tested the influence of spasmogen inhalation by **guinea pigs** upon subsequent demonstration of albumin-induced hyperreactivity in isolated airway tissues, such as perfused lung halves and tracheal spirals.

Corboz et al. (2000) used the isolated guinea pig lung to study the inhibition of capsaicin-induced bronchoconstriction by nociceptin. The lungs and the heart were removed en bloc from guinea pigs euthanized with an intraperitoneal overdose of sodium pentobarbital. The trachea and pulmonary artery were rapidly cannulated and half of the heart was cut to facilitate drainage. The lungs were then placed inside a warmed (37°C) glass chamber and suspended from a force displacement (Grass FT-03). They were mechanically ventilated with room air using a small animal ventilator. The respiratory rate was set at 60 strokes/min with a volume of 2.0 ml/stroke. Pulmonary inflation pressure was continuously monitored with a pressure transducer connected to a side arm of the tracheal can-

nula. Perfusion pressure was maintained with a peristaltic pump at a rate of 4.5–5.0 ml/min to produce a baseline pulmonary arterial pressure of between 6 and 14 cmH₂O. The pulmonary artery pressure was continuously monitored using a pressure transducer connected to the side arm of the pulmonary artery cannula. Transducers were connected to a polygraph for continuous monitoring of variables. The lungs were perfused with Tyrode's solution maintained at 37°C. Cumulative dose–response curves were constructed by adding increasing doses of capsaicin or the tachykinin NK₂ receptor agonist neurokinin A directly into the pulmonary artery. Test drugs were perfused 30 min before the addition of increasing doses of capsaicin or neurokinin A.

Anglade et al. (1998) measured the pulmonary capillary filtration coefficient in isolated **rabbit** lungs which were suspended by a string tied around the tracheal cannula from a counterbalanced force transducer to perform continuous weight measurement. To measure the pulmonary capillary filtration coefficient, pulmonary venous pressure was raised stepwise which results in an initial large weight gain for a few seconds followed by a slower rate of weight gain. The pulmonary capillary filtration coefficient was calculated on the slow phase of the weight curve by a time zero extrapolation or from the slope of the curve.

Nakamura et al. (1987) studied neurogenic pulmonary edema in lung perfusion preparations *in situ* in the **dog**.

Allen et al. (1993) studied the cardiovascular effects of a continuous prostacycline administration into an isolated *in situ* lung preparation in the dog.

Pogrebniak et al. (1994) investigated the influence of tumor necrosis factor in an isolated lung perfusion model in **pigs**.

The fluid filtration coefficient before and after infusion of *Escherichia coli* endotoxin was measured in excised **goat** lungs by Winn et al. (1988).

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D.2.2

In Vivo Tests

D.2.2.1

Bronchospasmolytic Activity in Anesthetized Guinea Pigs (Konzett–Rössler method)

PURPOSE AND RATIONALE

The principle was first described by Kiese (1935). Konzett and Rössler (1940) published a method suitable for screening procedures which found worldwide acceptance. A survey on the history and further modifications was given by Döring and Dehnert (1997).

The method is based on registration of air volume changes of a living animal in a closed system consisting of the respiration pump, of the trachea and the bronchi as well as of a reservoir permitting measurement of volume or pressure of excess air. Bronchospasm decreases the volume of inspired air and increases the volume of excess air. Thus, the degree

of bronchospasm can be quantified by recording the volume of excess air. Administration of spasmogens like acetylcholine, histamine, bradykinin, serotonin, ovalbumin, PAF, substance P, methacholine or leukotrienes, results in contraction of bronchial smooth muscle.

The method permits the evaluation of a drug's bronchospasmolytic effect by measuring the volume of air, which is not taken up by the lungs after bronchospasm.

PROCEDURE

Guinea-pigs of either sex weighing 250–500 g are anaesthetized with 1.25 g/kg i.p. urethane. Pentobarbital (60 mg/kg s.c.) and alcuronium chloride (1 mg/kg s.c.) are to be preferred when the bronchospasm is elicited by PAF or substance P. Anesthesia has to be deep enough in order to prevent influence of spontaneous respiration. The trachea is cannulated by means of a two way cannula, one arm of which is connected to the respiratory pump and the other to a Statham P23 Db transducer. The animal is artificially respired using a Starling pump with an inspiratory pressure set at 90–120 mm of water, an adequate tidal volume of 3 ml/100 g body weight and a frequency of 60 strokes per minute. Excess air, not taken up by the lungs, is measured and recorded on a polygraph. The internal jugular vein is cannulated for the administration of spasmogens and test compounds. The carotid artery is cannulated for measuring blood pressure.

Testing

Guinea-pigs receive the following spasmogens by i.v. administration:

- acetylcholine hydrochloride (20–40 µg/kg), or
- methacholine (20–40 µg/kg), or
- histamine dihydrochloride (5–20 µg/kg), or
- bradykinin triacetate (10–20 µg/kg), or
- ovalbumin (1 mg/kg), or
- PAF (25–50 ng/ml), or
- leukotrienes LTC₄, LTD₄ (about 1 µg/kg), or
- substance P (0.5 µg/kg).

After obtaining two bronchospasms of equal intensity, test compounds are administered i.v., p.o., s.c. or intraduodenally.

The spasmogen is given again at the following time intervals:

- 5, 15 and 30 min after i.v. administration of the drug
- 15, 30 and 60 min after intraduodenal administration of the drug
- 30 and 60 (sometimes also 120) min after p.o. administration of the drug.

The following standard compounds are used:

- atropine sulfate (0.01 mg/kg, i.v.) to inhibit acetylcholine or methacholine induced spasms
- aminophylline (6 mg/kg, i.v.) to inhibit bradykinin induced spasms
- tolpropamine-HCl (0.2 mg/kg) to inhibit histamine induced spasms
- imipramine-HCl (3–5 mg/kg) to inhibit serotonin-kreatinin-sulphate induced spasms.

EVALUATION

Results are expressed as percent inhibition of induced bronchospasm over the control agonistic responses. The ED_{50} value is calculated.

CRITICAL ASSESSMENT OF THE METHOD

The “Konzett-Roessler”-method has been proven to be a standard procedure in respiratory pharmacology being modified by several authors (Rosenthale and Dervinis 1968).

MODIFICATIONS OF THE METHOD

Forced insufflation was proposed as a simple but accurate inhalation procedure for investigating the activity of anti-asthmatic drugs in guinea pigs by Schiantarelli et al. (1982).

Lundberg et al. (1983), Belvisi et al. (1989), Miura et al. (1994) determined airway opening pressure (P_{ao}) as an index for tracheobronchial resistance to air flow.

Orr and Blair (1969) and Riley et al. (1987) sensitized rats intravenously with a potent antiserum to ovalbumin, obtained by infecting ovalbumin-sensitized donor rats with the parasitic nematode *Nippostrongylus brasiliensis* in order to boost IgE antibody production. The anesthetized animals were challenged with ovalbumin 48 h after sensitization and the subsequent increase in tracheal pressure was recorded.

Collier et al. (1993), Collier and James (1967) published a modification of the Konzett-Rössler-method using the forced re-inflation to overcome the severe bronchoconstriction occurring in sensitized guinea pigs.

Further modifications are by Schliep et al. (1986), Marano and Doria (1993).

Groeben and Brown (1996) measured changes in the cross-sectional area of conducting airways by cumulative doses of ipratropium with and without galamine, a selective M_2 muscarinic receptor blocker, and after metaproterenol in anesthetized dogs using high-resolution computed tomography. Using a Somatom Plus scanner (Siemens), 50 to 55 contiguous

scans were obtained, starting approximately 5 mm above the origin of the right upper lobe bronchus from the trachea and proceeding caudally using 1-mm table feed and 2-mm slice thickness. The dogs were anesthetized with thiopental. After paralysis was induced by succinylcholine, the trachea was intubated and the lungs ventilated with a volume-cycled ventilator with 100% oxygen. During the scans, the dogs were apneic at function residual capacity (approximately 2 min). Images were reconstructed using a high-spatial frequency algorithm.

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D.2.2.2**Effect of Arachidonic Acid or PAF on Respiratory Function In Vivo****PURPOSE AND RATIONALE**

Based on the classical method of Konzett and Rössler (1940), Lefort and Vargafting (1978), Vargafting et al. (1979) studied the effects of arachidonic acid and PAF on respiratory function of guinea pigs *in vivo*.

Arachidonic acid is metabolized into thromboxane (TXA₂) and prostacyclin (PGI₂). TXA₂ produced in the lung leads to bronchoconstriction, which is independent from circulating platelets and leukotrienes; TXA₂ produced intracellularly in platelets induces a reversible thrombocytopenia. PGI₂ produced in the vessel wall leads to the reduction of systolic and diastolic blood pressure. All three effects are inhibited by drugs which block cyclo-oxygenase. In contrast, agents which block thromboxane synthetase inhibit bronchoconstriction and thrombocytopenia, but lead to a potentiation of blood pressure reduction.

In contrast to arachidonic acid, PAF as inducer leads to bronchoconstriction, which is platelet-dependent. In addition, PAF induces thrombocytopenia, leukocytopenia, reduction of blood pressure and increase of hematocrit. These effects are also reversible, but more persistent than those induced by arachidonic acid, and quickly result in tachyphylaxis. The test allows to evaluate the sites of action of drugs, which interfere with the mechanisms of bronchoconstriction and thrombocytopenia; in an *in vivo*-model guinea pigs are challenged with the spasmogens and platelet-aggregating substances arachidonic acid or PAF (platelet activating factor).

PROCEDURE

Male guinea pigs (Pirbright White) weighing 300–600 g are anesthetized with 60 mg/kg pentobarbital

sodium (i.p.). One of the jugular veins is cannulated for the administration of spasmogen and test compound. Both external carotid arteries are cannulated; one is connected to a pressure transducer to register blood pressure, the other is used for blood withdrawal. The trachea is connected to a Starling pump with an inspiratory pressure set of 80 mm H₂O, an adequate tidal volume of approx. 10 ml/kg body weight and a frequency of 70–75 strokes/min. Spontaneous respiration is inhibited by intravenous injection of pancuronium (4 mg/kg) or gallamine (2 mg/kg) on time.

In some experiments, pulmonary β -receptors are blocked by intraperitoneal administration of propranolol (2 mg/kg).

Excess air, not taken up by the lungs, is conducted to a transducer with bronchotimer (Rhema, Germany) which translates changes in air flow to an electrical signal. Changes in air flow and arterial blood pressure are recorded continuously.

Animals receive multiple intravenous injections of the same dose of arachidonic acid (Sigma, 250–600 μ g/kg prepared from a stock solution 10 mg/ml ethanol, 1:20 dilution with Na₂CO₃) until two bronchospasms of equal intensity are obtained. The test compound is administered intravenously and the spasmogen is given again at the following intervals: 2, 10, 20 and, if necessary, 30 min after administration of the drug.

Ordinarily, the lung has to be passively dilated (bronchotimer) after each of the bronchoconstrictions. Immediately before and 30–45 s after each of the arachidonic acid applications, approx. 50 μ l blood are collected into Na-EDTA-coated tubes. The number of thrombocytes is determined with a platelet analyzer (Becton Dickinson Ultra-Flo-100 or Baker 810) in 10 μ l samples of whole blood.

PAF (Paf-acether C16, Bachem, 0.03–0.04 μ g/kg in 0.9% saline+0.1% human serum albumin) as inducer is injected intravenously 60 min before, 5 min and, if necessary, 60 min after intravenous drug administration. Blood samples are collected 30 s before and 15 s after each of the PAF applications. The number of leukocytes and hematocrit values are determined automatically (TOA-microcell counter CC 108, Colora Meßtechnik).

Standard compounds:

- dazoxiben HCl (inhibitor of thromboxane synthetase, TSI)
- acetylsalicylic acid (inhibitor of cyclo-oxygenase, COI)

EVALUATION

Percent inhibition or increase of bronchospasm, reduction of blood pressure, thrombocytopenia, leukocytopenia and hematocrit following test drug administration are calculated in comparison to control values before drug treatment. For the reduction of blood pressure, both the magnitude [mm Hg, systolic and diastolic] and the duration [min] are determined. Even a sole increase in duration of blood pressure reduction is considered as an increase of the effect. From the pattern profile of the influence on bronchoconstriction, thrombocytopenia and blood pressure reduction, the mechanism of action of a test drug is concluded:

- inhibitor of thromboxane synthetase
- inhibitor of cyclo-oxygenase
- other effect = no profile

In addition, the inherent action of the test substance on blood pressure is determined before arachidonic acid- or PAF-administration.

MODIFICATIONS OF THE METHODS

Kagoshima et al. (1997) used a modification of the Konzett-Rössler-method to test the suppressive effects of a PAF-antagonist on asthmatic responses in guinea pigs actively sensitized with ovalbumin. The immediate and the late asthmatic response were measured by the oscillation method according to Mead (1960).

Pauluhn (1994, 2004) described inhalation systems, mainly for toxicological studies, by which large numbers of animals can be studied simultaneously.

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D.2.2.3

Bronchial Hyperreactivity

PURPOSE AND RATIONALE

Symptoms like asphyctic convulsions resembling bronchial asthma in patients can be induced by inhalation of histamine or other bronchospasm inducing agents in guinea pigs. The challenging agents are applied as aerosols produced by an ultra-sound nebulizer. The first symptoms are increased breathing frequency, forced inspiration, and finally asphyctic convulsions. The occurrence of these symptoms can be delayed by antagonistic drugs. Pre-convulsion time, i. e. time until asphyctic convulsions, can be measured.

PROCEDURE

Ten male albino guinea pigs weighing 300–400 g per group are used. The inhalation cages consist of 3 boxes each ventilated with an air flow of 1.5 l/min. The animal is placed into box A to which the test drug or the standard is applied using an ultra-sound nebulizer LKB NB108 which provides an aerosol of 0.2 ml solution of the test drug injected by an infusion pump within 1 min. Alternatively, the animal is treated orally or subcutaneously with the test drug or the standard. Box B serves as a sluice through which the animal is passed into box C. There, the guinea pig is exposed to an aerosol of a 0.1% solution of histamine hydrochloride provided by an ultra-sound nebulizer (De Vilbiss, Model 35 A). Time until appearance of asphyctic convulsions is measured. Then, the animal is immediately withdrawn from the inhalation box. The aerosols are removed from the back wall of the boxes by applying low pressure.

EVALUATION

Percent of increase of pre-convulsion time is calculated versus controls. ED_{50} values can be found, i. e. 50% of increase of pre-convulsion time.

CRITICAL ASSESSMENT OF THE METHOD

The “guinea pig asthma” has been applied as useful method in various modifications by many laboratories.

MODIFICATIONS OF THE METHODS

Simple methods to test bronchospasmolytic activity in conscious animals, called “thoracography” were described by Herxheimer (1956) Olsson (1971), Beume et al. (1985). A silicon tube with a diameter of 1 mm is filled with mercury and serves as strain gauge applied as belt around the thorax of conscious guinea pigs. Changes in electrical resistance due to breathing

movements of the thorax are registered. The animals are exposed in a Plexiglas chamber to acetylcholine nebulized by an ultrasonic device. Time until onset of coughing indicated by an increase of signal amplitude and of severe asthmatic dyspnoea is registered.

Immunological factors are involved in bronchial hyperreactivity (Reynolds 1991).

Harris et al. (1976) immunized rabbits with thermophilic actinomyces antigen (*Micropolyspora faeni*). Lesions resembling hypersensitivity in man were found, characterized by a mononuclear cell interstitial reaction and a marked increase in the number of intra-alveolar cells.

Ufkes et al. (1983) induced bronchial and cardiovascular anaphylaxis in Brown-Norway rats, sensitized with trinitrophenyl haptenized ovalbumin and AlPO_4 as adjuvant 12 days prior to challenge with trinitrophenyl haptenized bovine serum albumin intravenously.

Raeburn et al. (1992) gave a survey on techniques for drug delivery to the airways and the assessment of lung functions in animal models including parameters such as lung compliance and airway resistance.

Elwood et al. (1992) studied the effects of dexamethasone and cyclosporin A on the airway hyperresponsiveness and the influx of inflammatory cells into bronchoalveolar lavage fluid seen 18 to 24 h after exposure to aerosolized ovalbumin in actively ovalbumin-sensitized Brown-Norway rats.

Schmiedl et al. (2003) found an increase of inactive intra-alveolar surfactant subtypes in lungs of asthmatic Brown Norway rats. The volume fractions of surfactant subtypes and the epithelial surface fraction covered with alveolar edema were determined by point and intersection counting. The surface activity of surfactant from broncho-alveolar lavage was determined as the minimum surface tension at minimal bubble size with a pulsating bubble surfactometer.

Pahl et al. (2002) and Kuss et al. (2003) exposed ovalbumin-sensitized Brown Norway rats to an ovalbumin-containing aerosol in a nose-only inhalation system (TSE, Bad Homburg, Germany) for 1 h to provoke an influx of inflammatory cells into the airways. At the time of maximal influx of eosinophilic granulocytes into the airways (48 h later), the animals were sacrificed and a broncho-alveolar lavage was performed.

A model of bronchial hyperreactivity after active anaphylactic shock in conscious guinea pigs has been described by Tarayre et al. (1990). The guinea pigs were sensitized by an intramuscular injection of a large dose of ovalbumin in Freund's adjuvant. The adminis-

tration of ovalbumin to induce anaphylactic shock was by aerosol. Bronchial hyperreactivity to histamine was observed 3–6 h after the anaphylactic shock.

Bolser et al. (1995) sensitized guinea pigs by intraperitoneal injection of 200 $\mu\text{g}/\text{kg}$ ovalbumin mixed with 200 mg/kg aluminum hydroxide. The animals were placed 28 days later in a transparent plastic chamber and exposed to aerosols of ovalbumin (0.1%–1%) at an airflow of 4 l/min to elicit coughing. Coughs were detected by a microphone placed in the chamber and connected to an audio monitor and chart recorder. The number of coughs elicited during a 4-min exposure was counted by visual inspection of the chart record.

Pons et al. (2000) described a guinea pig model of asthma. Animals were sensitized by two intraperitoneal injections of 20 μg ovalbumin and 100 mg aluminum hydroxide given 24 h apart. The sensitized animals were anesthetized and mechanically ventilated. Air flow was measured with a pneumotachograph, along with transpulmonary pressure and arterial pressure. Lung resistance and dynamic compliance were calculated. Then, 30 min after administration of test drug, the animals were challenged by inhaled ovalbumin (5 mg/ml).

The importance of eosinophil activation for the development of allergen-induced bronchial hyperreactivity was underlined by Santing et al. (1994). A significant increase in bronchoreactivity to histamine was observed at 6 h after allergen exposure, which was associated with an increase of eosinophils in the bronchoalveolar lavage and an increase in the eosinophil peroxidase activity.

The increased pulmonary vascular permeability may be related to the adult respiratory distress syndrome in man (Snapper and Christman 1989).

The infusion of small amounts of *Escherichia coli* endotoxin into chronically instrumented awake **sheep** results in well-characterized pulmonary dysfunction (Brigham and Meyrick 1986).

One animal model associated with both increased airway responsiveness and pulmonary inflammation is endotoxemia in sheep (Hutchinson et al. 1983).

The effect of a platelet activating factor receptor antagonist on the sheep's response to endotoxin was studied by Christman et al. (1987).

Chiba and Misawa (1995) characterized muscarinic cholinergic receptors in airways of antigen-induced airway hyperresponsive rats. The animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract together with *Bordetella pertussis* (2×10^6) as an adjuvant and were boosted with 2,4-dinitrophenylated *Ascaris suum* extract 5 days later. Isometric contractions of the circu-

lar muscle of isolated bronchial rings after addition of increasing doses of acetylcholine were measured with a force-displacement transducer.

Laboratory infection of **primates** with *Ascaris suum* may provide a model of allergic bronchoconstriction (Patterson et al. 1983; Pritchard et al. 1983; Eady 1986).

Richards et al. (1986) used various models of airway hypersensitivity, such as inhalation of *Ascaris suum* antigen in monkeys and dogs.

Rylander and Marchat (1988) studied the effect of a corticosteroid on an acute inflammation in the lungs of guinea pigs exposed to an aerosol of bacterial endotoxin. The subsequent inflammatory response was evaluated counting the number of cells obtained from airway lavage and in the lung interstitium as well as the chemotactic effect of alveolar macrophages.

Hatzelmann et al. (1996) reported on automatic leukocyte differentiation in broncho-alveolar lavage fluids of guinea pigs and Brown-Norway rats using an automatic cell analyzing system (Cobas Helios 5Diff; Hoffmann-La Roche; Grenzach-Wyhlen, Germany).

Minshall et al. (1993) demonstrated that neonatal immunization of **rabbits** with *Alternaria tenuis* can lead to the development of persistent airway hyperresponsiveness.

Okada et al. (1995) studied late asthmatic reactions in **guinea pigs** sensitized with *Ascaris* antigen. They evaluated interleukin-1 production by immunostaining with anti-IL-1 β antibody and elucidated the action of IL-1 in late asthmatic reactions with recombinant IL-1 receptor antagonist.

Folkerts et al. (1995) found that intratracheal inoculation of *parainfluenza type 3 virus* to guinea pigs induces a marked increase of airway responsiveness to increasing doses of intravenous histamine. In spontaneously anesthetized breathing guinea pigs, inhalation of an aerosol containing the nitric oxide precursor L-arginine completely prevented the virus-induced hyperresponsiveness to histamine.

Fryer et al. (1994, 1997) measured M₂ muscarinic receptor function in anesthetized and paralyzed guinea pigs by electrical stimulation of both vagus nerves producing bronchoconstriction (measured as pulmonary inflation pressure) and bradycardia.

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flectory increase of respiratory frequency after histamine inhalation are attenuated by bronchodilatory drugs. Additional respiratory parameters can be recorded using a Fleisch tube and a catheter inserted into the pleural cavity (Englert et al. 1992). The method can be used for various purposes, e. g., to evaluate the antagonism against bradykinin-induced bronchoconstriction (Wirth et al. 1991, 1993) or the bronchodilator effects of potassium channel openers (Englert et al. 1992) or to measure the effect of morphine on respiration in rats (Kokka et al. 1965).

PROCEDURE

Guinea pigs of either sex weighing 400–600 g are anesthetized with 70 mg/kg pentobarbital i.p. The trachea, pleural cavity, jugular vein, and carotid artery are prepared and cannulated. The animals are mechanically ventilated with a Starling respiratory pump which delivers an inspiration volume that represents a tracheal pressure of 8 cm water at a rate of 60 strokes/min. Succinylcholine chloride at a dose of 1 mg/kg is given i.v. to prevent interference from spontaneous respiration. The guinea pigs are placed inside a whole body plethysmograph and tracheal, pleural, venous and arterial catheters are connected to onset ports in the wall of the plethysmograph box. The tracheal port is then connected with the respiration pump. **Airflow rate** into and out of the plethysmograph are measured as pressure difference with a No. 000 Fleisch tube and a differential pressure transducer (Fa. Hellige, PM 97 TC). Airflow is calibrated by passing compressed air through a rotameter. The **tidal volume** (V_T) is calculated from the flow signal. **Transpulmonary pressure** (P_{TP}) is measured with a differential pressure transducer (Hellige, PM 97 TC), with one side attached to a catheter inserted into the right pleural cavity and the other side connected to a side port of the tracheal cannula. P_{TP} is calibrated with a water manometer. Signals from airflow, tidal volume and transpulmonary pressure are fed into an on-line computer system (PO-NE-MAH, Model PF-1, Storrs) for calculation of **pulmonary resistance** (R_L) and **dynamic lung compliance** (C_{DYN}). These parameters are calculated for each breath with a sampling rate of 100 s⁻¹ for each circle. Flow and pressure signals for computation are obtained from a PLUGYS measuring system (Fa. Hugo Sachs Elektronik, Freiburg, Germany). Systemic arterial pressure is measured using a Statham pressure transducer (P 23 Db). Heart rate is computed from pressure pulses (Döring and Dehnert 1997).

Three doses of test compound or standard are injected intravenously. Saline injections serve as con-

D.2.2.4

Body Plethysmography and Respiratory Parameters After Histamine-Induced Bronchoconstriction in Anesthetized Guinea Pigs

PURPOSE AND RATIONALE

Guinea pigs can be placed in a plethysmograph for measurement of respiratory parameters. Respiratory frequency and respiratory amplitude are recorded. The decrease of respiratory amplitude (diminished respiratory volume due to bronchoconstriction) and the re-

trols. Intravenous injections of histamine (0.5–2 µg/kg) lead to a short decrease in C_{DYN} and to a short increase in R_L by approximately 200% compared with baseline. Challenges are repeated at 5-min intervals, yielding the same increase in R_L during the whole 1-h experimental period. After 3 reproducible responses the test agent is administered intravenously 1 min before the histamine injection.

To evaluate test compounds for inhalation route, aerosols are generated with an ultrasonic nebulizer (LKB, model NB 108) and are administered to the animals through a shunt in the afferent limb of the respiratory pump, allowing the inspired air to pass through the nebulizer chamber before entering the animals lungs.

EVALUATION

Inhibition of histamine induced bronchoconstriction by various doses of test compound and standard is recorded. ED_{50} values for inhibition in R_L are calculated. Furthermore, the time course of histamine antagonism can be evaluated. Compounds can be tested either after iv. injection of histamine (prevention) or during intravenous infusion of histamine (intervention).

CRITICAL ASSESSMENT OF THE METHOD

Whole body plethysmography has been proven to be a useful tool in respiratory pharmacology for studies on the antagonism against various bronchoconstrictors, such as histamine and bradykinin, as well as for airway pharmacology of potassium channel openers.

MODIFICATIONS OF THE METHOD

Several authors use body plethysmography to study respiratory functions in animals (Amdur and Mead 1958; Blümcke et al. 1967), Pennock et al. 1979; Agarwal (1981), James and Infesto (1983), Kisagawa et al. (1984), Griffith-Johnson (1988), Danko and Chapman (1988), Ball et al. (1991), Chand et al. (1993).

The effect of β -blockers on pulmonary function and bronchoconstrictor responsiveness in **guinea pigs** and rats has been studied by Chapman et al. (1985).

Finney and Forsberg (1994) developed a technique for quantification of nasal involvement in a guinea pig plethysmograph. Nasal and lower respiratory system conductance could be measured simultaneously in anesthetized animals.

A whole body plethysmograph for conscious animals has been described by Elliott et al. (1991) and improvements by Linton (1991).

Studies of bronchospasmolytic agents with aerosol challenge in conscious guinea pigs using a double

chamber plethysmograph box have been reported by Schlegelmilch (1991).

Ball et al. (1991) described a method for the evaluation of bronchoactive agents in the conscious guinea pig. The method involves the use of "head out" whole body plethysmographs from which respiratory rate can be recorded by monitoring respiration-related changes in pressure within the body chamber.

Hey et al. (1995) used a head-out, whole body plethysmograph to examine the effects of GABA_B receptor agonists on minute ventilation, tidal volume and respiratory rate due to room air and carbon dioxide-enriched gas hyperventilation in conscious guinea pigs.

Murphy et al. (1998) developed a method for chronic measurement of pleural pressure in conscious **rats**. Pleural pressures were measured by surgically implanting a fluid-filled polyurethane catheter attached to a pressure-sensitive radiotelemetry transmitter (Model TA11PA-C40, Data Sciences Int., St. Paul, MN) beneath the pleural surface. A compatible receiver (Model RLA0120) and a data acquisition and analysis software system (LabPRO, Version 3) sampling at a rate of 500 Hz were used to analyze the telemetric signals.

Sinnett et al. (1981) described a fast integrated flow plethysmograph for small animals (**mouse**).

Glaab et al. (2001) used tidal mid-expiratory flow in a head-out body plethysmograph as a measure of airway hyperresponsiveness in allergic mice.

Simple methods to determine respiratory parameters in small animals were described by Schütz (1960), Höbel et al. (1971).

Schlenker (1984) and Schlenker and Metz (1989) evaluated ventilatory parameters in dystrophic **Syrian hamsters**.

Wasserman and Griffin (1977) studied bronchoactivity in the intact anesthetized **dog**.

Paré et al. (1976) determined pulmonary resistance and dynamic lung compliance in **rhesus monkeys**.

Wegner et al. (1984) measured dynamic respiratory mechanics in monkeys by forced oscillations generated by a loudspeaker in an airtight chamber.

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D.2.2.5

Pneumotachography in Anesthetized Guinea Pigs

PURPOSE AND RATIONALE

The use of a pneumotachograph based on the principle of the Fleisch-tube and of additional pressure transducers allows simultaneous measurements of several respiratory and circulatory parameters in anesthetized guinea pigs (de la Motta 1991).

PROCEDURE

Guinea pigs (Pirbright white) weighing 300–400 g are anesthetized with 1.5 g/kg urethane i.p. The animals are shaved ventrally at the neck, placed on a heated operating table, and fixed at the upper extremities. A metal cannula with a blunted tip is inserted into the trachea and secured with a loop within the caudal section of the trachea. A thin plastic catheter is inserted into the esophagus and the tip located inside the thorax in order to register intrathoracic pressure. Furthermore, the cephalic vein at one side and the carotid artery on the opposite side are cannulated. The tracheal cannula is connected with pieces of tubing to a Fleisch tube (pneumotachograph), size 0000. In or-

der to avoid water condensation, the Fleisch tube is heated. The Fleisch tube is connected with a sensitive differential pressure transducer with a range of 2 cm H₂O (Validyne, model MP 45 -xx-871). One side of another pressure differential transducer with a range of 20 ml H₂O is connected with the esophageal catheter, the other side remaining open to room air. Both Validyne pressure transducers are connected to a separate preamplifier. For recording of the arterial blood pressure a Gould pressure transducer, Type P23Gb, is used. The signals for airflow and esophageal pressure are monitored at the output of the preamplifier with a digital 2-channel oscilloscope. To obtain various respiratory and circulatory parameters from the three primary signals they are calculated by certain formulae by an analogue computer (Buxco Pulmonary Mechanics Analyzer, Model 6). The following parameters are presented at the output of the instrument as analogue electrical signals:

Circulation

Systolic blood pressure, diastolic blood pressure, mean blood pressure

Respiration

Tidal volume, respiratory volume per minute, respiratory rate

Pulmonary Mechanics

Airway resistance, dynamic compliance, end-respiratory work.

A multi-channel recorder (Graphtec Linearorder Mark VII) serves a functional check on the Buxco Analyzer and as analogue presentation of the calculated parameters. Data processing is performed by a 12-channel A/D converter (Buxco Data Logger, Model D/C-12 F/V) which digitizes the analogue output signals of the Buxco Analyzer and sends them through a serial interface (RS232) to an IBM PC. A special software program (Lomask 1987; Hastings 1990a, b) provides a flexible facility for data reduction and statistical evaluation.

EVALUATION

For each individual experiment the data of the last 5 min before the first substance application are averaged and used as controls. The response values after substance application are then expressed as percentages of the controls. In this way each animal serves as its own control. For an analysis of the results the response values are averaged over certain time intervals.

CRITICAL ASSESSMENT OF THE METHOD

The method described in great detail by de la Motta (1991) may be modified using different equipment according to individual needs.

MODIFICATIONS OF THE METHOD

Measurement of respiratory parameters is based on earlier studies to be mentioned for historical reasons (Gad 1880; Pflüger 1882; Zwaardemaker and Ouwehand 1904; Jaquet 1908; Rohrer 1915; Gildemeister 1922; Fleisch 1925; v. Neergaard and Wirz 1927; survey by Döring HJ 1991).

Lorino et al. (1988) assessed respiratory mechanics of histamine bronchopulmonary reactivity in guinea pigs.

O'Neil et al. (1981) published a comparative study of respiratory responses to bronchoactive agents in rhesus and cynomolgus monkeys.

Rayburn et al. (1989) described a computer-controlled pulmonary function system for studies in large animals.

Five methods of analyzing respiratory pressure-volume curves have been compared by Lai and Diamond (1986).

A specially designed pneumotachograph that is placed inside the trachea of guinea pigs was described by Santing et al. (1992) allowing the evaluation of airway functions in conscious, unstressed animals.

Lorino et al. (1993) estimated the changes in end-respiratory lung volume accompanied histamine-induced bronchoconstriction in anesthetized, paralyzed, and mechanically ventilated guinea pigs from measurements of thoracic cross-sectional area, assessed from the voltage induced by an external uniform magnetic field in a pickup coil encircling the rib cage.

Gozzard et al. (1996) evaluated the effects of PDE-inhibitors in **New Zealand White rabbits** which were immunized within 24 h of birth with *Alternaria tenuis* antigen. Spontaneously breathing rabbits were intubated in neuroleptanalgesia with a cuffed endotracheal tube connected to a thermoregulated Fleisch pneumotachograph to allow measurement of tidal air flow. An oesophageal balloon catheter was inserted to provide a measure of intra-pleural pressure and transpulmonary pressure. Antigen challenge was performed with inhaled *Alternaria tenuis* extract.

An excellent survey on various methods and equipment to measure air flow is given by Döring and Dehnert (1997).

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D.2.2.6

Airway Microvascular Leakage

PURPOSE AND RATIONALE

Plasma exudation in guinea-pig airways *in vivo* can be determined by Evans Blue dye and is fairly correlated with radiolabelled albumin (Rogers et al. 1989). This method can be used to study the antagonism against bradykinin- and platelet-activating factor-induced airway microvascular leakage and vagal stimulation-induced airway responses (Sakamoto et al. 1992, 1994).

PROCEDURE

Female Dunkin-Hartley guinea pigs weighing 380–600 g are anesthetized with an initial dose of 1.5 g/kg urethane injected i.p. Additional urethane is given i.v. 30 min later to achieve an appropriate level of anesthesia. A tracheal cannula is inserted into the lumen of the cervical trachea, a polyethylene catheter into the left carotid artery to monitor blood pressure and heart rate and another polyethylene catheter into the external jugular vein for administration of drugs. The animals are connected to a constant volume mechanical ventilator and then given an injection of 1.0–1.5 mg/kg suxamethonium i.v. to prevent interference with spontaneous respiration. A tidal volume of 10 ml/kg and a frequency of 60 strokes/min are used.

Lung resistance is measured as an index of airway function and monitored throughout the experiment. Transpulmonary pressure is measured with a pressure transducer with one side attached to a catheter inserted into the right pleural cavity and the other side attached to the side port of the intratracheal cannula. Airflow is measured by a pneumotachograph connected to a pressure transducer. The signals of the transducers are used for instantaneous calculation of lung resistance by an appropriate computer program.

The test compound (bradykinin receptor antagonist) is given intravenously. Ten min later, Evans Blue dye (20 mg/ml) is injected i.v. for 1 min. After 1 min, bronchoconstriction and microvascular leakage is induced by injection of bradykinin or by inhalation of bradykinin or PAF or vagal stimulation.

Six min after induction of leakage, the thoracic cavity is opened, and a cannula is inserted into the

aorta through a ventriculotomy. Perfusion is performed with 100–150 ml 0.9% saline at a pressure of 100–120 mm Hg in order to remove the intravascular dye from the systematic circulation. Blood and perfusion liquid are expelled through an incision in the right and left atrium. Subsequently, the right ventricle is opened, and the pulmonary circulation is perfused with 30 ml of 0.9% saline. The lungs are then removed, and the connective tissue, vasculature, and parenchyma are gently scraped. The airways are divided into 4 components: lower part of the trachea, main bronchi, the proximal 5 mm portion, and the distal intrapulmonary airways. The tissues are blotted dry, and then weighed. Evans Blue dye is extracted in 2 ml of formamide at 40°C for 24 h, and measured in a spectrophotometer at 620 nm.

EVALUATION

Evans Blue dye concentration, expressed as ng/mg tissue, as well as lung resistance are compared by statistical means (unpaired Student's *t*-test or Mann-Whitney U test) between treated groups and controls receiving the challenge only.

MODIFICATIONS OF THE METHOD

Boschetto et al. (1989) tested the effect of antiasthma drugs on microvascular leakage in guinea pig airways. Microvascular leakage was induced by intravenous injection of platelet-activating factor (50 ng/kg) which acts directly on venular endothelial cells, and measured by quantifying extravasation of Evans blue dye.

Xu et al. (1998) induced pulmonary edema in rats by injection of 20 µg/kg angiotensin I and studied the suppression by ACE-inhibitors, ATII antagonists and α-adrenergic receptor blockers.

Rapidly developing pulmonary edema was induced by intravenous injection of 1.2 mg/kg of the GABA agonist bicuculline in rats and the role of endogenous endothelin was examined by Herbst et al. (1995).

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D.2.2.7

Isolated Larynx In Situ

PURPOSE AND RATIONALE

The *in situ* isolated larynx of rats has been recommended by Willette et al. (1987) for evaluation peripheral opiate receptor antagonists. Peripheral opioid-induced laryngospasm and central opioid-induced respiratory depression can be measured simultaneously. Fentanyl citrate stimulates both peripheral and central opiate receptors, whereas [D-Ala²-Met⁵]-enkephalinamide stimulates only peripheral opiate receptors. Compounds that inhibit both laryngeal and respiratory effects of fentanyl, e. g., naloxone HCl, can be considered both central and peripheral opiate antagonists. Compounds that inhibit only the peripheral effects of fentanyl, e. g., naltrexone methylbromide, can be considered peripheral opiate receptor antagonists.

PROCEDURE

Laryngeal resistance experiments are carried out in male Sprague Dawley rats weighing 340–360 g and anesthetized with urethane (900 mg/kg, i.p.) The animals are secured in supine position and the left femoral artery and the left and right femoral veins are cannulated for recording of arterial blood pressure and administration of drugs. A right-angle polyethylene cannula (ID 1.67 mm, OD 2.42 mm) is inserted into the caudal trachea at the level of the manubrium for measuring tracheal flow via a small animal pneumotachograph. Care has to be taken to avoid damaging surrounding blood vessels or adjacent bilateral laryngeal nerves. Tracheal air flow is continually sampled (300 ml/min) for the breath to breath analysis of end tidal carbon dioxide with an infrared gas analyzer.

Laryngeal resistance in the rat is determined using a modification of the methods described by Stransky et al. (1973), Bartlett et al. (1973), and Willette et al. (1982b). The rostral portion of the trachea is cannulated with a right-angle polyethylene tube (ID 1.67 mm, OD 2.42 mm). This cannula is carefully advanced towards the larynx and secured with a suture (4–0 silk). A constant flow ($V=30$ ml/min) of compressed air, maintained with a flow meter, is delivered through a cannula. Pre-laryngeal pressure is measured with

a needle tipped pressure transducer inserted into the lumen of the flow cannula. A 1.75-cm segment of polyethylene tubing (OD 8.4 mm, ID 4.6 mm) is placed into the mouth to retract resistive components in the nasopharyngeal region.

Laryngeal resistance (LR) is calculated by the following equation:

$$LR = (P_L P_1 - P_L P_0) / V$$

where $P_L P_1$ is the laryngeal pressure in the cannula directing a constant flow (V) through the larynx. $P_L P_0$ is the pressure in the cannula when it is removed from the trachea.

The agonist fentanyl is administered through the left femoral vein at a dose of 12 $\mu\text{g}/\text{kg}$ which is equivalent to 1.5 times the ED_{99} in the conscious rat tail flick assay. The enkephaline analogue [D-Ala²-Met⁵]-enkephalinamide is injected at a dose of 250 $\mu\text{g}/\text{kg}$ which acts peripherally and increases laryngeal resistance (Willette 1982a). The opiate receptor antagonists are administered similarly into the right femoral vein.

At the conclusion of the experiment, the pulmonary afferent stimulant, phenyldiguanide (25 $\mu\text{g}/\text{kg}$, i.v.) is injected into the right femoral vein to elicit laryngospasm and to determine the viability of the preparation.

EVALUATION

All summary values are expressed as the mean plus or minus the standard error of the mean (SEM) Comparisons are made with independent and paired two-tailed t -tests.

MODIFICATIONS OF THE METHOD

Inagi et al. (1998) assessed the effect of botulin toxin in the rat larynx by measurement of the optical density of PAS-stained laryngeal muscle after electrical stimulation, spontaneous laryngeal muscle activity, and laryngeal movement.

O'Halloran et al. (1994) studied the effects of upper airway cooling and CO_2 on breathing and on laryngeal and supraglottic resistance in anesthetized rats.

González-Barón et al. (1989) studied the modifications of larynx resistance changing bronchial tone in cats evoked by intravenous administration of 10 $\mu\text{g}/\text{kg}$ carbachol as bronchoconstrictor and by fenterol (10 $\mu\text{g}/\text{kg}$) or isoproterenol (0.1 mg/kg) as bronchodilators.

Wang et al. (1999) developed an isolated, luminally perfused laryngeal preparation in anesthetized paralyzed cats in order to compare the effects of solutions

with varying levels of pH and pCO_2 on pressure-sensitive laryngeal receptor sensitivity.

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D.2.2.8

Animal Models of Asthma

D.2.2.8.1

Treatment of Asthma

PURPOSE AND RATIONALE

Several authors described asthma models in mice. Hammad et al. (2004) found in a mouse model of asthma that activation of peroxisome proliferator-activated receptor- γ (PPAR- γ) in dendritic cells inhibits the development of eosinophilic airway inflammation.

PROCEDURE

Bone Marrow Dendritic Cells

OVA-TCR transgenic mice (DO10.11) on a BALB/c background, bred at the Erasmus University (Rotterdam) (Murphy et al. 1990), are used. Femurs and tibiae of female mice are removed and flushed with RPMI 1640. Vigorous pipetting disintegrates clusters within the marrow suspension. The cells are washed, enumerated, and plated in Petri dishes. Cell-culture medium (TCM) is supplemented with gentamicin (60 $\mu\text{g}/\text{ml}$), 2-mercaptoethanol (5×10^{-5} mol/l) and 5% fetal calf serum (Lutz et al. 1999). At day 0 of the culture, cells are seeded at a concentration of 2×10^6 /dish in medium containing granulocyte-macrophage colony-

stimulating factor (GM-CSF, 200 IU/ml). At days 6 and 8, half of the medium is collected, centrifuged, and the pellet is resuspended in TCM containing 200 IU/ml of recombinant murine GM-CSF.

At day 9 of the culture, dendritic cells (Banchereau and Steinman 1998) are pulsed overnight with ovalbumin containing the vehicle (dimethylsulfoxide, DMSO) in which the standard PPAR- γ agonist (e. g., rosiglitazone) is suspended or with medium alone as control. Other plates are treated with the test drug or a PPAR- γ antagonist. After antigen pulsing overnight, non-adherent dendritic cells are collected, washed to remove free ovalbumin, and resuspended in phosphate-buffered saline at a concentration of 12.5×10^6 cells/ml.

Eosinophilic Airway Inflammation

For intratracheal injection of dendritic cells (Lambrecht et al. 2000), mice are anesthetized with Avertin and 80 μ l of the cell suspension (1×10^6 dendritic cells) is instilled through the opening vocal cords. Mice are injected with unpulsed dendritic cells, ovalbumin-treated dendritic cells or dendritic cells treated additionally with the PPAR- γ antagonist. From days 10–13, mice are exposed to 30-min ovalbumin aerosols. They are sacrificed 24 h after the last aerosol. Broncho-alveolar lavage is performed with 3×1 ml of Ca^{2+} - and Mg^{2+} -free buffer supplemented with 0.1 mmol/l sodium EDTA. The broncho-alveolar lavage is centrifuged; the cells are resuspended in buffer and enumerated with a hemocytometer. They are differentiated (Vremec and Shortman 1997) by staining for 30 min with anti-I-Ad/I-Ed FITC (macrophages), anti-CCR3 PE (eosinophils), anti-CD3-cy-chrome, anti-B220 cytochrome (T and B cells, respectively) and anti-CD11c APC (macrophages) in PBS containing 0.5 bovine serum albumin and 0.01% sodium azide. Cells are washed and analyzed by flow cytometry (Van Rijt et al. 2002).

EVALUATION

The difference between the groups is calculated using the Mann–Whitney *U*-test for unpaired data.

MODIFICATIONS OF THE METHOD

Iwasaki et al. (2001) recommended atopic NC/Nga mice as a model for allergic asthma: after immunization with ovalbumin, severe allergic responses were elicited by a single intranasal challenge.

For testing PPAR agonists, Trifilieff et al. (2003) used a murine model of asthma using lung inflammation induced by ovalbumin or by LPS (Trifilieff et al.

2000). Female BALB/c mice (4 weeks old) were immunized with ovalbumin on days 0 and 14, exposed to an aerosol challenge of ovalbumin or phosphate-buffered saline (PBS) on day 21 and killed on day 23 for measurement of inflammatory cells in the broncho-alveolar lavage. Animals were intranasally treated with compounds 1 h before the aerosol exposure using PBS containing 2% DMSAO as vehicle.

For LPS-induced lung inflammation mice were challenged intranasally with 0.3 mg/kg of LPS (*Salmonella typhosa*) in 50 ml of sterile PBS or with sterile PBS alone and killed 3 h later for broncho-alveolar lavage. Tumor necrosis factor alpha (TNF- α) and the neutrophil chemokine KC levels were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, UK). Animals were intranasally treated with compounds 1 h before the aerosol exposure using PBS containing 2% DMSO as vehicle.

Mueller et al. (2003) reported that **peroxisome proliferators-activated receptor γ ligands** attenuate immunological symptoms of allergic asthma. For asthma induction, Balb/c mice were injected with 10 μ g of ovalbumin in Alum twice on days 1 and 5 (Zhang et al. 1999). On day 10, they were challenged intranasally with 40 μ g ovalbumin in sterile saline, every day for 3 days. On the day after the last intranasal challenge, the mice were sacrificed. The degree of inflammation in the lung was evaluated by histopathology. Gene expression was tested by microarray analysis, and serum IgE determined by ELISA.

Regal et al. (2001) described trimellitic anhydride-induced eosinophilia in a mouse model of occupational asthma. Trimellitic anhydride was conjugated to mouse serum albumin. Female BALB/c mice were sensitized on days 1 and 3 intradermally with 0.1 ml of 0.3% ovalbumin suspended in corn oil. On day 12, animals were additionally sensitized intratracheally with 0.04 ml ovalbumin, trimellitic anhydride conjugated to mouse serum albumin, or mouse serum albumin dissolved in water. For elicitation of the allergic response, mice were challenged intratracheally beginning on day 19 with 0.04 ml of aqueous solutions of ovalbumin, trimellitic anhydride conjugated to mouse serum albumin, or mouse serum albumin under ketamine/xylazine anesthesia. After the last intratracheal instillation, the mice were anesthetized with pentobarbital, EDTA plasma was collected by cardiac puncture, the trachea was cannulated and the lungs were lavaged with two 0.9-ml aliquots of PBS to obtain bronchial lavage fluid. The lungs were removed for homogenization and analysis of eosinophil peroxidase and myeloperoxidase.

Churg et al. (2001) studied anti-inflammatory effects of alpha-1 antitrypsin and a metalloprotease inhibitor in C57 BL/6 mice or macrophage metalloelastase knockout mice after intratracheal instillation of a single 7-mg dose of crystalline silica (α -quartz, Minusil-5; US Silica Corporation, Clarkstown, W.Va., USA). This dose produced a rapid and persisting acute inflammatory infiltrate. Mice were euthanized 2 or 24 h after dust exposure by halothane overdose and the lungs removed from the chest cavity. A 20-gauge catheter was inserted into the trachea and the lungs lavaged six times with 1 ml of ice-cold saline. For inflammatory cell measurements, the saline lavage was centrifuged at 200 g at 4°C for 10 min. The supernatants were decanted, and the cell pellets were resuspended in 200 μ l of saline. Total cell counts were performed using a hemocytometer and differential cell counts were performed on a 10- μ l drop of the cell suspension heat-fixed on a slide and stained with hematoxylin-eosin. Lavage samples were analyzed for desmosine and hydroxyproline.

De Sanctis and Drazen (1997) discussed the genetics of native airway responsiveness in mice.

Several animal species have been used such as **rats**.

Misawa et al. (1987) found strain differences among Wistar, Lewis and Fischer 344 rats in an allergic asthma model.

Misawa and Sugiyama (1993) described an airway hyperresponsiveness model in rats, inducing allergic asthma with DNP-*Ascaris* extract.

Uhlig et al. (1998) reported the effects of long-term oral treatment with leflunomide on allergic sensitization, lymphocyte activation, and airway inflammation in rats.

Birrell et al. (2003) investigated the nitric-oxide synthase isoform involved in eosinophilic inflammation in a rat model of Sephadex-induced airway inflammation. The rat model of Sephadex-induced airway inflammation was also used by Belvisi et al. (2005) for preclinical studies on ciclesonide, an inhaled corticosteroid for the treatment of asthma.

Many authors used sensitized **Brown Norway rats** in experimental models of asthma (Elwood et al. 1992; Steerenberg et al. 1999; Nonaka et al. 2000; Xu et al. 2000; Blesa et al. 2002; Glaab et al. 2002; Huang et al. 2002; Belvisi et al. 2005; Valstar et al. 2006).

Guinea pigs have been used as animal models for asthma by many authors. Most studies were performed with sensitization either by injection with ovalbumin suspended in an adjuvant (Santives et al. 1976; Banner et al. 1996; Lawrence et al. 1998; Regal et al. 2000; Li et al. 2001; Santing et al. 2001; Mukaiyama et al.

2004; Boskabady and Zarei 2004; Ikezono et al. 2005; Tang et al. 2005) or by repetitive sensitization with inhaled ovalbumin (Sagara et al. 1996, 1997; Smith et al. 1996; Liu et al. 1997; Tohda et al. 1998; Cheng et al. 2001; Zhang et al. 2002) and then challenge by inhaled ovalbumin.

Fujimura et al. (1997) described a guinea pig model of ultrasonically nebulized distilled-water-induced bronchoconstriction. Guinea pigs sensitized by intradermal injection of ovalbumin in Freund's adjuvant were treated with aerosols of physiological saline generated by an ultrasonic nebulizer.

Zhou et al. (1998) reported a dose-response relationship between exposure to cockroach allergens and induction of sensitization in an experimental asthma in Hartley guinea pigs.

Larsen and Regal (2002) studied trimellitic anhydride dust-induced airway obstruction and eosinophilia in non-sensitized guinea pigs.

Nishitsuji et al. (2004) described a guinea pig model of cough variant asthma. Bronchial responsiveness to methacholine and cough reflex sensitivity to capsaicin were measured 72 h after ovalbumin inhalation in actively sensitized guinea pigs.

Dogs were used as animal models for asthma by several authors. Many studies preferred the **Basenji Greyhound dog**, which manifests various characteristics of human asthma, including airway hypersensitivity to low concentrations of methacholine (Hirshman et al. 1980; Hirshman and Downes 1981; Chan et al. 1985; Darowski et al. 1989; Emala et al. 1993, 1996).

Redman et al. (2001) studied pulmonary immunity to ragweed in a **Beagle dog** model of allergic asthma.

Some studies were reported using **cats** as an animal model for asthma. Norris et al. (2003) and Norris-Reinero et al. (2004) described an experimental model of allergic asthma in cats sensitized to the house dust mite and Bermuda grass allergen.

Several studies were performed using **monkeys** as animal models for asthma. *Ascaris suum*-sensitive monkeys were used by Mauser et al. (1995) and Zou et al. (2002). Rhesus monkeys (*Macaca mulatta*) were used by Patterson et al. (1975) and Patterson and Harris (1981, 1990, 1992). Turner et al. (1996) used *Macaca fascicularis* to demonstrate *in vitro* and *in vivo* effects of leukotriene B₄ antagonism in primates.

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D.2.2.8.2**Prevention of Allergic Asthma Reaction****PURPOSE AND RATIONALE**

Type 1 allergy is an abnormal reaction to protein substances that occur naturally. B-lymphocytes produce an antibody against the allergen. An allergic reaction occurs every time the body is exposed to the allergen causing release of histamine. Allergic reactions can be asthma, hay fever or nettle rash. Sensitization to normally harmless environmental antigens (e. g., pollen, house-dust mite) is the prerequisite for initiating the inflammatory cascade in bronchial asthma. Inflammation of the airway mucosa is orchestrated by Th-2 type T cells, which produce Th-2 cytokines (IL-4, IL-5, IL-9, IL-13, and IL-15), which regulate both IgE production and airway inflammation. At present, no cure of asthma disease is available, but primary and secondary prevention is the therapy of choice (Cieslewicz et al. 1999). Specific immunotherapy is performed by injection of increasing amounts of allergens to induce hyporesponsiveness to the respective allergen. By mucosal application of soluble antigen, mucosal tolerance can be achieved (van Halteren et al. 1997; Astori et al. 2000). Many studies have been performed in spite of the fact that no animal model is available that resembles all features of human bronchial asthma (Herz et al. 1998). In particular, models using recombinant allergens were described (Hoyne et al. 1997, 2000; Herz et al. 2004).

These studies concern specific allergens, such as **ovalbumin** (Renz et al. 1992; Marth et al. 2000; Neuhaus-Steinmetz et al. 2000; Raap et al. 2003; Reader et al. 2003; Wegmann et al. 2005), **mite dust allergen** (Hoyne et al. 1996; Clarke et al. 1999; Yasue et al. 1998a, 1998b, 1998c, 1999; Jarnicki and Thomas 2002), **pollen allergen** (Hirahara et al. 1998; Wiederemann et al. 1999, 2001; Batanero et al. 2002; Repa et al. 2004; Hufnagl et al. 2005; Winkler et al. 2006), **latex protein allergen** (Thakker et al. 1999; Woolhiser et al. 2000; Meade and Woolhiser 2002; Hufnagl et al. 2003), **bee venom allergen** (Von Garnier et al. 2000), and **cat allergen** (Briner et al. 1993; Treter and Luqman 2000).

Wegmann et al. (2005) studied involvement of distal airways in a chronic model of experimental asthma.

PROCEDURE**Animals**

Pathogen-free female BALB/c mice (Harlan Winkelmann, Hannover, Germany), weighing 18–22 g and 6–8 weeks of age, were used in all experiments. The

animals were maintained under standard housing conditions, fed an ovalbumin- (OVA-) free diet and supplied with food and water ad libitum.

Sensitization Protocol

Mice were sensitized to OVA by three intraperitoneal injections [10 µg OVA grade VI (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg Al(OH)₃ diluted in 200 µl phosphate-buffered saline (PBS)] on days 1, 14, and 21. The mice were challenged with OVA (grade V) aerosol (1% wt/vol in PBS) via the airways twice a week on two consecutive days over a period of 12 weeks (Renz et al. 1992). Sham sensitization and challenges were carried out with sterile Al(OH)₃ in PBS. Animals were analyzed after 1 or 12 weeks of OVA aerosol challenge. To investigate persistence of airway inflammation and lung physiological changes mice were analyzed after 6 weeks of OVA aerosol challenge discontinuation following 12 weeks of OVA aerosol challenge.

**Differential Cell Counts
in Broncho-Alveolar Lavage Fluid**

At 48 h after the last allergen challenge broncho-alveolar lavage (BAL) was performed and analyzed (Renz et al. 1992).

Measurement of Cytokines in BAL Fluid

IL-4, IL-5 and tumor necrosis factor alpha (TNF- α) were measured in cell-free lavage fluids by cytometric bead array (CBA, BD Biosciences, San Diego, Calif., USA). The detection limit for each of the cytokines was 10 pg/ml. Complete BAL transforming growth factor beta (TGF- β) was measured after acidic activation using chicken anti-human TGF- β in a standard ELISA protocol. The detection limit for TGF- β was 20 pg/ml.

Lung Histology

Lungs were fixed *ex situ* with 4% (wt/vol) paraformaldehyde via the trachea, removed and stored in 4% paraformaldehyde. Lung tissues were embedded into paraffin, and 3 µm sections were stained with haematoxylin and eosin or periodic acid-Schiff staining. For localization of collagen fibrils, Sirius red staining was performed according to Uhal et al. (1998).

Immunohistochemistry

Indirect immunohistochemistry was used to stain lung sections for smooth muscle cells and myofibroblasts (mouse monoclonal IgG against α -SMA, clone 1A4, Immunotech, Marseilles, France), and nerve cells

(polyclonal rabbit antiserum against protein gene product 9.5, Biogenesis, Poole, UK) according to Fehrenbach et al. (2002). To distinguish smooth muscle cells from myofibroblasts a rat monoclonal IgG against fibroblast-specific peptide (clone ER-TR7; Biogenesis) was utilized.

Quantitative Morphology

Paraffin sections stained with Fast Green/Sirius Red and for α -SMA, respectively, were used to quantify changes in airway epithelial cell, collagen and smooth muscle cell layers of distal airways according to standard stereological methods. A distal airway was defined as the segment of a terminal bronchiolus that, starting at the broncho-alveolar duct transition, extended up to five alveoli along the proximal direction. Using a PC-based Olympus light microscope BX 51 equipped with a CAST-Grid System (Visiopharm, Hoersholm, Denmark), all distal airways of a given section were delineated (at a magnification of $\times 426$), and the fields of view to be analyzed (at a final magnification of $\times 1.700$) were automatically defined according to systematic uniform random sampling. The arithmetic mean thicknesses were determined as the volume of the respective component, determined by counting all points hitting airway epithelium, Sirius Red- and α -SMA-positive components, respectively. Results were referred to the reference surface determined by counting all intersections with the airway epithelial basal membrane (Reader et al. 2003). The arithmetic mean thicknesses were calculated.

Electron Microscopy and Definition of Proximal and Distal Airways

For analysis of airway wall ultrastructure, lungs of mice chronically challenged with OVA and control mice challenged with PBS ($n = 3$ per group) were fixed by instillation of 4% (wt/vol) paraformaldehyde via the trachea. Beginning at the lobar bronchus, the airways were microdissected along the axial pathway according to Plopper et al. (2001). For histopathological and ultrastructural analysis, proximal airways were defined as the lobar bronchi and the axial pathway down to the fourth intrapulmonary branch point. Mid-level airways were defined as the axial pathways between the intrapulmonary branch points 6–12. Terminal bronchioles with direct connection to the alveolar ducts were defined as distal airways (Postlethwait et al. 2000).

Non-invasive Measurement of Mid-Expiratory Airflow at Baseline and of Bronchial Responsiveness to Methacholine

The mid-expiratory airflow (EF₅₀) was measured 24 h after the last OVA aerosol challenge using head-out body plethysmography (Glaab et al. 2001).

EVALUATION

Results are presented as mean values \pm SD unless stated otherwise. One-way ANOVA test or Student's unpaired *t*-test was used to determine the significance of differences between animal groups.

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D.2.2.9

Bleomycin-Induced Pulmonary Fibrosis

PURPOSE AND RATIONALE

Pulmonary fibrosis has been induced by bleomycin in various species: golden Syrian **hamsters** (Giri et al. 1986; Chen et al. 1997; Gurujeyalakshmi et al. 1999; Iyer et al. 1999, 2000), **mice** (Taooka et al. 1997; Tamagawa et al. 2000; Keerthisingam et al. 2001; Terasaki et al. 2000; Terasaki 2001; Atzori et al. 2004) and **rats** (Howell et al. 2001; Simler et al. 2002; Wang et al. 2002; Morcillo and Bulbena 2003).

Iyer et al. (1999) determined the effects of pirfenidone on procollagen gene expression at the transcription level in a bleomycin hamster model of lung fibrosis.

PROCEDURE

Treatment of Animals

Male golden Syrian hamsters weighing 90–110 g were housed in groups of four in facilities with filtered air and constant temperature and humidity. A 12-h/12-h light/dark cycle was maintained, and the animals had access to water and either pulverized Rodent Laboratory Chow 5001 or the same pulverized chow containing pirfenidone (0.5% w/w). The hamsters were fed these diets 2 days before intratracheal instillation and throughout the study period. Hamsters were instilled intratracheally with saline or bleomycin (7.5 units/kg per 5 ml). The animals were sacrificed at 3, 7, 10, 14, and 21 days after the bleomycin or saline instillation by decapitation, and their lungs were removed and freeze-clamped, dropped in liquid nitrogen, and stored at -80°C . The major portion of the sample was used for direct total RNA isolation, and the remainder was used for other biochemical studies.

Tissue Processing for Biochemical Study

The frozen lungs were thawed and homogenized in 0.1 M KCl/0.02 M Tris-HCl buffer, pH 7.6, with a Polytron homogenizer. After recording the total homogenate volume (5–6 ml), it was mixed, divided into aliquots, and stored at -80°C , except for the aliquots for lipid peroxidation and hydroxyproline assays, which were processed and assayed the same day on which the lungs were homogenized.

Determination of Lipid Peroxidation

The lung malondialdehyde equivalent (MDAE) level, an index of lipid peroxidation, was determined in the whole homogenate according to the method of Ohkawa et al. (1979)

Determination of Prolyl Hydroxylase Activity

The method for prolyl hydroxylase assay is based on the release of tritiated water from 3,4- ^3H proline-labeled unhydroxylated procollagen substrate prepared *in vitro* using 10-day-old embryonic chick tibiae (Giri et al. 1983). During the reaction, tritium is released in stoichiometric proportion to prolyl hydroxylation as tritiated water, which is collected and counted as a measure of the prolyl hydroxylase activity (Hutton et al. 1966). The activity was expressed as the total dpm released/lung per 30 min.

Determination of Prolyl Hydroxylase Activity In Vitro

Control hamsters not subjected to any treatment were first anesthetized with sodium pentobarbital (80–100 mg/kg). Their lungs were perfused with ice-cold isotonic saline; then, all lung lobes were dissected out and rinsed in saline. They were immediately homogenized in buffer containing 0.1 M Tris and 0.05% Triton X. The homogenate was spun down at 6000 g for 20 min at 4°C . The supernatant was gently aspirated and used to determine prolyl hydroxylase activity and its protein content. The procedure to measure the prolyl hydroxylase activity was essentially the same as described above. Briefly, the reaction mixture in a total volume of 2.2 ml consisted of 200 μl of α -ketoglutarate (0.001 M), 200 μl of ferrous ammonium sulfate (0.005 M), 250 μl of supernatant as enzyme source, 200 μl of pirfenidone to produce the desired final concentration, 200 μl of Tris-HCl (1 M), and 20 μl of ^3H -unhydroxylated PC substrate (400,000 cpm). The reaction mixture was first preincubated with pirfenidone at different concentrations for 30 min at 37°C in a shaking water bath. The reaction was started by adding 200 μl of ascorbic acid (0.005 M); 30 min later, the reaction was terminated by adding 200 μl of 50% trichloroacetic acid. The tritiated water released was collected by vacuum distillation. Then 1 ml of the tritiated water was mixed with 10 ml of Ready Safe liquid scintillation cocktail (Beckman), and the radioactivity was determined at 45% counting efficiency in a scintillation counter. The protein content of the supernatant was determined according to the method of Lowry et al. (1951). The prolyl hydroxylase activity was expressed as total dpm associated with tritiated water released in the reaction mixture/mg protein per 30 min.

Determination of Hydroxyproline

For assay of lung hydroxyproline as a measure of collagen content, 1 ml of whole homogenate was precipitated with 0.25 ml of ice-cold 50% (w/v) trichloroacetic acid and centrifuged, and the precipitate was hydrolyzed in 2 ml of 6 N HCl for 18 h at 110°C . The hydroxyproline content was measured according to the method of Woessner (1961).

EVALUATION

All data are expressed as mean \pm SEM. Bleomycin treatment increases the amount of proteins of extrapulmonary origin that can result in the artificial lowering of all values (Karlinski and Goldstein 1980; Goldstein and Fine 1986). Thus, the *in vivo* data are expressed on a per-lung basis. The data were compared within the groups at the corresponding times using the two-way

ANOVA where four groups were involved, and the *t* test between the two groups. A value of $P \leq 0.05$ was considered to be the minimum level of statistical significance.

MODIFICATIONS OF THE METHOD

Howell et al. (2001) induced pulmonary fibrosis in **rats**. Male Lewis rats, aged 6 weeks and weighing 140–170 g, were anesthetized by intramuscular injection of 0.75–1.0 ml/kg Hypnorm (fentanyl citrate, 0.315 mg/ml, and fluanisone, 10 mg/ml). Bleomycin sulfate was administered by a single intratracheal injection (1.5 mg/kg body weight in 0.3 ml of sterile saline). Control animals received 0.3 ml of saline alone. In initial experiments, groups of six rats were killed by pentobarbitone overdose after 6 days to allow assessment of thrombin levels in broncho-alveolar lavage fluid. Separate groups of two rats were sacrificed 1, 3, 6, and 14 days after bleomycin instillation for immunohistochemical assessment of thrombin and protease-activated receptor1. Lungs were fixed by intratracheal instillation of 4% paraformaldehyde, the trachea ligated, and the inflated lungs and heart removed *en bloc*. Tissues were fixed and transferred to 15% sucrose in phosphate-buffered saline, before alcohol dehydration and embedding in paraffin wax. An additional series of animals was killed 6 days after bleomycin or saline instillation for measurement of blood coagulation parameters, total and differential cell counts in broncho-alveolar lavage fluid, and for Northern blot analysis of lung tissue connective tissue growth factor and $\alpha_1(I)$ procollagen mRNA levels. For measurement of coagulation parameters, blood was collected from the inferior vena cava of animals after laparotomy, and was immediately mixed 10:1 with a solution of 3.8% trisodium citrate (w/v). For measurement of total lung collagen and connective tissue growth factor and procollagen mRNA levels, the vasculature was perfused with 5 ml of sterile saline containing 100 U/ml heparin. The lungs were removed, weighed, and immediately snap-frozen in liquid N₂ after removing the trachea and major airways.

Adachi et al. (2003) and Azoulay et al. (2003) studied the effects of granulocyte colony-stimulating factor (G-CSF) on lung injury induced by various doses of bleomycin in rats.

Sogu et al. (2004) found that endosteine, an antioxidant, prevents bleomycin-induced pulmonary fibrosis in rats.

Chen et al. (2006) reported that short courses of low-dose dexamethasone delay bleomycin-induced lung fibrosis in rats.

Chaudhary et al. (2006) described the time course of inflammation and fibrosis in the rat bleomycin model and studied the effect of timing of anti-inflammatory and antifibrotic treatments on efficacy.

Barrio et al. (2006) studied *in vitro* tracheal hyperresponsiveness to muscarinic receptor stimulation by carbachol in the rat model of bleomycin-induced pulmonary fibrosis.

Terasaki et al. (2000) and Terasaki (2001) studied the effect of epimorphin in bleomycin-induced pulmonary fibrosis in **mouse**. Pulmonary fibrosis was induced in 8-week-old male ICR mice by a single intratracheal instillation of bleomycin. On selected days after injection (days 0, 3, 7, 14, 21, 28, 35, 42, and 56), the lungs were harvested and investigated. Immunohistochemical analysis was performed for epimorphin using light microscopy and confocal microscopy and also the analysis by immunoelectron microscopy and *in situ* hybridization.

Avivi-Green et al. (2006) reported that discoidin domain receptor 1-deficient mice are resistant to bleomycin-induced lung fibrosis. Matsuyama et al. (2006) found that suppression of discoidin domain receptor 1 by RNA interference attenuates lung inflammation induced by intratracheal instillation of bleomycin in mice.

Inayama et al. (2006) reported that a $I\kappa B$ kinase- β inhibitor ameliorates bleomycin-induced pulmonary fibrosis in mice.

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D.2.2.10

Influence of Cytokines on Lung Fibrosis

PURPOSE AND RATIONALE

Several cytokines (Murphy et al. 2000) are involved in development of pulmonary fibrosis, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF α), and transforming growth factor beta (TGF β) (Kelly et al. 2003; Agostini and Gurrieri 2006).

GM-CSF is involved in fibrotic reactions of the lung (Xing et al. 1996; Adachi et al. 2002, 2003).

TGF β is a central modulator of pulmonary and airway inflammation and fibrosis (Sime et al. 1997; Kolb et al. 2002; Gauldie et al. 2003; Xu et al. 2003; Yao et al. 2004; Hardie et al. 2006; Lee et al. 2006; Shepard 2006).

Smad proteins are involved in the TGF β -mediated pulmonary fibrosis (Zhao et al. 2000; Bonniaud et al. 2004, 2005; Kobayashi et al. 2006).

TNF α induces TGF β (Sime et al. 1998; Warshamana et al. 2001; Sullivan et al. 2005).

Xing et al. (1996) reported that the **transfer of GM-CSF gene** to rat lung induces eosinophilia, monocytosis, and fibrotic reactions.

PROCEDURE

Construction of Recombinant Adenovirus Vectors

An 800-bp fragment of murine GM-CSF cDNA was isolated from pCDSR α by digestion with *Bam*HI and *Dra*I. The shuttle plasmid pACCMV contain-

ing 0 to 17 mu human type 5 adenovirus genome with a CMV promoter (760 bp), multicloning sites and SV40 splicing junction/polyA signal (430 bp) inserted in the E1 region of viral genome was first digested with *SaI*I, and the ends were repaired using T4 kinase and dNTPs (New England Biolabs, Beverly, Mass., USA), followed by a secondary digestion with *Bam*HI to generate the 3' complimentary ends. The GM-CSF fragment was then subcloned into the *Bam*HI/*SaI*I site in PACCMV using T4 ligase (New England Biolabs) to generate the recombinant plasmid PACCMVmGM-CSF. The presence of the GM-CSF insert was confirmed by restriction digestions. The PACCMVmGM-CSF was co-transfected, following a standard procedure described previously (Graham and Prevec 1991), into 293 cells along with a plasmid pAdBHG10 which contained the most rightward sequences (3.7 to 100 mu) of human type 5 adenovirus genome with a partial deletion in the E3 region (Bett et al. 1994). The recombinant replication-deficient adenovirus Ad5E1PACCMVmGM-CSF (Ad5E1GM-CSF) was rescued by homologous recombination. The presence of GM-CSF cDNA in the viral genome was verified by analyzing viral genome fragments upon *Hind*III digestion and by Southern hybridization (Graham and Perc 1991). The control virus Ad5dl70-3 was constructed and characterized as previously described (Bett et al. 1994), and, similar to Ad5E1GM-CSF, this virus had the E1 region crippled, hence incapable of replication.

High titers of the above viruses were generated (Graham and Prevec 1991). Briefly, viruses purified by two rounds of CsCl gradient centrifugation were subjected to chromatography using PD-10 Sephadex columns (Pharmacia Biotech, Baie d'UrFe, Quebec, Canada) to remove CsCl. The virus fractions were collected in PBS containing 10% glycerol, measured for conductance to ensure complete removal of CsCl, pooled, titered, aliquoted and stored at -70°C until use.

Characterization of Recombinant Adenovirus Vector Expressing GM-CSF In Vitro

GM-CSF transgene mRNA was examined by Northern hybridization analysis (Xing et al. 1993) using total RNA from 293 cells infected with 10 plaque-forming units (pfu)/cell of Ad5E1GM-CSF for 24 and 48 h. The supernatants from these cells and from infected rat alveolar macrophages were assayed for GM-CSF using an ELISA kit (Endogene, Cambridge, Mass., USA). This ELISA was specific for mouse GM-CSF without crossreactivity with rat GM-CSF, with a sensitivity of 4 pg/ml.

Delivery of Recombinant Adenovirus Vectors to the Lung

Following a standard procedure (Xing et al. 1994), 300 μl of Ad5E1GM-CSF or control virus Ad5dl70-3 diluted in PBS to a concentration of 1×10^9 pfu was instilled intratracheally to the lung of Sprague Dawley male rats weighing 220–280 g (Charles River Laboratories, Ottawa, Canada). At the end of 1, 2, 4, 7, 12, 18, and 24 days after gene transfer, rats were anesthetized, blood samples were taken from the abdominal aorta, and serum preparation and bronchoalveolar lavage (BAL) were performed.

Transgene and Transgene Protein Expression in the Lung

The left lung of rats obtained at each time point was snap-frozen in liquid nitrogen. Total lung RNA extraction and Northern hybridization were performed. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine transgene mRNA expression with total lung RNA using PCR reagents from Promega (Madison, Wis., USA) following the standard protocol. The specific primers for PCR were chosen to ensure the amplification of the transgene-specific GM-CSF mRNA but not the endogenous rat GM-CSF mRNA (Miyatake et al. 1985; Smith et al. 1994). The sense and anti-sense primer sequences were 5'-GTCTCTAACGAGTTCTCCTTCAAG-3' and 5'-TTCAGAGGGCTATACTGCCTTCCA-3', respectively. The primers for rat GAPDH were designed as described by Rosenfeld et al. (1992). BAL samples were collected at various times and assayed for transgene protein GM-CSF by ELISA as described above or for TNF α by ELISA specific for both murine and rat TNF α (Genzyme, Cambridge, Mass., USA). Serum samples collected from the same animals were also assayed for circulating levels of GM-CSF by ELISA.

Cytologic Examination of BAL and Blood Samples

Total cell numbers in BAL were determined using a hemacytometer. Differential cell types were determined on cytopins stained with Diff-Quik (Baxter, McGaw Park, Ill., USA) by randomly counting 300–400 cells/cytopin. To analyze the total peripheral blood leukocyte counts and differentials, blood samples were collected into heparin-coated tubes. Total leukocyte numbers were counted on a hemacytometer after lysing red blood cells with a lysis buffer containing 94% H₂O, 3% acetic acid, and 3% Diff-Quik purple stain. Differential leukocytes were determined on blood smears stained with Diff-Quik by counting 500–700 cells/blood smear.

Histopathologic Examination of Lung and other Tissues

The right lung and in some instances the whole lung of each animal were fixed by perfusion with 10% formalin (Fisher Scientific, Fairlawn, N.J., USA). Tissues from heart, liver, spleen, and kidney were also fixed in 10% formalin. Multiple sections from different lobes of the lung or from other organs were stained with hematoxylin/eosin for routine histopathology, with Congo Red for identification of tissue eosinophils, or with Elastic van Gieson for collagen and elastin.

MODIFICATIONS OF THE METHOD

Underwood et al. (2000) demonstrated in guinea pigs and rats that a p38 MAPK inhibitor reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung.

Kolb et al. (2001) reported that the proteoglycans decorin and biglycan differentially modulate TGF- β -mediated fibrotic responses in the lung of mice.

Uhal et al. (2003) showed that in rats amiodarone induced lung fibrosis and alveolitis, which could be partially inhibited by angiotensin system antagonists.

Jiang et al. (2004) described regulation of pulmonary fibrosis by the chemokine receptor CXCR3, which is the receptor for the interferon- γ -inducible C-X-C chemokines MIG/CXCL9, IP-10/CXCL10, and ITAC/CXCL11.

Kim et al. (2006) tested the alveolar epithelial-to-mesenchymal cell transition, which develops *in vivo* during pulmonary fibrosis, and found regulation by the extracellular matrix.

Shi-Wen et al. (2006) found that constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis and gave evidence of an autocrine endothelin loop operating through the endothelin A and B receptors.

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D.2.2.11

Emphysema Models

PURPOSE AND RATIONALE

Several animal models of genetically determined emphysema are known, such as the **tight-skin mouse** (Rossi et al. 1984; Martorana et al. 1989; Gayraud et al. 2000), beige mutants of the C57BL6J strain (O'Donnell et al. 1999; Lucattelli et al. 2003), the **blotchy mouse** (Ranga and Kleinerman 1981; McCartney et al. 1988), and **homozygous mutant *klotho* (*KL*^{-/-}) mice** (Suga et al. 2000).

Hoyle et al. (1999) described emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor. Le Cras et al. (2004) reported that vascular endothelial growth factor causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice. Tsa et al. (2004) reported that overexpression of placenta growth factor contributes to the pathogenesis of pulmonary emphysema in mice.

Blanco et al. (1989), Whitney et al. (1999), Massaro et al. (1995), Massaro and Massaro (2000, 2003), and Dirami et al. (2004) reported that in **rats** septation of gas-exchange sacculi occurs during the first two postnatal weeks. Treatment with dexamethasone irreversibly impairs septation. Treatment with all-*trans*-retinoic acid prevents the dexamethasone-induced inhibition of septation.

Hind and Maden (2004) and Maden and Hind (2004) found that retinoic acid induces alveolar regeneration in the adult mouse lung after damage by disulphiram treatment. These data could not be confirmed in an elastase-induced emphysema model by Fujita et al. (2004).

Several authors used elastase instillation to produce emphysema-like lesions in **rats** (Tepper et al. 2000; March et al. 2004), **mice** (Inoue et al. 2003; Murakami et al. 2005), **rabbis** (Qi et al. 2004), and **dogs** (Morino et al. 2005).

Kuraki et al. (2002) described inhibition of human neutrophil elastase-induced emphysema in rats by an oral neutrophil elastase inhibitor.

PROCEDURE

Rat Emphysema Model

Male Wistar rats weighing 228 ± 15 g were divided into controls (saline treated), low-dose group (treated with 200 U human neutrophil elastase), and a high-

dose group (treated with 400 U human neutrophil elastase). Human neutrophil elastase was sprayed above the carina of the trachea using a microsyringe without tracheotomy. Eight weeks after human neutrophil elastase application, rats were sacrificed and lungs were dissected out to evaluate the morphologic changes.

Effects of Neutrophil Elastase Inhibitor

Rats were divided into four groups: saline control, human neutrophil elastase + CMC, human neutrophil elastase + low dose of inhibitor, and human neutrophil elastase + high dose of inhibitor given orally. Six hours after human neutrophil elastase, lung hemorrhage and neutrophil accumulation in the lung were determined. Eight weeks after the application, the functional and morphologic changes were determined.

Lung Hemorrhage and Neutrophil Accumulation in the Lung

After tracheostomy, broncho-alveolar lavage was performed for determination of neutrophil counts and hemoglobin in lavage fluid. In addition, neutrophil accumulation was estimated using the myeloperoxidase activity.

Lung Volume and Pulmonary Mechanics

Eight weeks after human neutrophil elastase application, rats were anesthetized and tracheotomy was performed. Using a whole-body plethysmograph for small animals, the functional residual capacity (FRC), total lung capacity and static lung compliance were determined.

EVALUATION

Data were presented as mean \pm SD. Differences between groups were evaluated for statistical significance using one-way analysis of variance.

MODIFICATIONS OF THE METHOD

Boström et al. (1996) showed that platelet-derived growth factor A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis.

Kirschvink et al. (2005) induced production of pulmonary MMP-2 and MMP-9 and emphysema by repeated cadmium nebulizations in rats.

Corteling et al. (2002) studied the migration and activation of neutrophils into the airway in pathological conditions such as pulmonary emphysema in BALB/c and C57BL/6 mice and in golden hamsters. The animals were sequentially treated intranasally with 0.3 mg/kg lipopolysaccharide and with 0.5 mg/kg N-formyl-Met-Leu-Phe.

Selman et al. (2003) induced emphysema in guinea pigs by exposure to the whole smoke of 20 cigarettes per day, 5 days per week, for 1 month, 2 months, and 4 months through a whole-body exposure chamber. Half of the animals received a matrix metalloproteinase inhibitor. After death, the lungs were lavaged with saline solution, and matrix metalloproteinases in the lavage fluid were determined by zymography and immunoblot. Lungs were fixed for histology, immunohistochemistry, and morphometry.

Lucattelli et al. (2003) described collagen phagocytosis by lung alveolar macrophages in animal models of emphysema.

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D.2.2.12

Models of Chronic Obstructive Pulmonary Disease (COPD)

PURPOSE AND RATIONALE

Chronic obstructive pulmonary disease (COPD) is a severe respiratory condition that is increasing in prevalence worldwide. The disease is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with abnormal inflammatory response of the lungs to noxious particles and gases. Three conditions comprise COPD, namely mucus hypersecretion, emphysema and bronchiolitis. Cigarette smoking is the major risk factor for development of COPD and accounts for the majority of cases (Donnelly and Rogers 2003). The relevance of the present animal models for COPD has been questioned (Canning 2003). Several authors used chronic cigarette-smoke exposure in mice (Hautamaki et al. 1997; Cavarra et al. 2001a, 2001b; Wright and Churg 2002; Bartalesi et al. 2005; Martorana et al. 2005), in rats (Escolar et al. 1995; Lee et al. 2005), in guinea pigs (Wright 2001; Meshi et al. 2002), or in dogs (Frasca et al. 1983).

Lee et al. (2005) reported inhibition of cigarette-smoking-induced emphysema and pulmonary hypertension in rat lungs by a HMG-CoA reductase inhibitor.

Martorana et al. (2005) found that a selective phosphodiesterase-4 (PDE4) inhibitor fully prevents emphysema in mice chronically exposed to cigarette smoke.

PROCEDURE

Six-week-old C57Bl/6J male mice were, in acute studies, exposed either to room air or the smoke of five cigarettes (Virginia filter cigarettes: 12 mg of tar and 0.9 mg of nicotine) for 20 min. In chronic studies, the mice were exposed to either room air or to the smoke of three cigarettes/day for 5 days/week for 7 months. Antioxidant capacity was assessed at the end of exposure in broncho-alveolar lavage fluid. Cytokines and chemokines were determined.

In acute studies, mice were divided in three groups of 40 animals each. These groups were then divided

into four subgroups of 10 mice: no treatment/air exposed; no treatment/smoke exposed; low dose of test compound/smoke exposed; high dose of test compound/smoke exposed.

In chronic studies, five groups of animals were used: no treatment/air exposed; drug treatment/air exposed; no treatment/smoke exposed; low dose of test compound/smoke exposed; high dose of test compound/smoke exposed. After 7 months, animals were sacrificed and the lungs fixed intratracheally with 5% formalin at a pressure of 20 cmH₂O. Lung volume was measured by water displacement. Assessment of emphysema included mean linear intercept and internal surface area. The volume density of macrophages, marked immunohistochemically with antimouse Mac-3 monoclonal antibodies, was determined by point counting.

EVALUATION

The significance of the differences was calculated using one-way analysis of variance.

MODIFICATIONS OF THE METHOD

Kumar et al. (2003) compared a selective PDE4 inhibitor with pentoxifylline (a non-selective phosphodiesterase inhibitor) and dexamethasone in ameliorating the lesions of chronic asthma in BALB/c mice sensitized to ovalbumin and chronically challenged with aerosolized antigen for 6 weeks.

Kodavanti et al. (2000) reported that the combination of elastase and sulfur dioxide exposure causes COPD-like lesions in the rat.

The potential of tachykinin receptor antagonists in airways diseases was discussed by Joos and Pauwels (2001).

Sturton and Fitzgerald (2002) reviewed PDE4 inhibitors for the treatment of COPD.

Billah et al. (2002) described the pharmacology of an orally active PDE4 inhibitor.

Pitfalls and opportunities for modeling allergic asthma in mice were discussed by Kumar and Foster (2002).

Inoue et al. (2003) described an impaired pulmonary inflammation response as a prominent feature of streptococcal pneumonia in mice with experimental emphysema.

Dual dopamine D₂ receptor and β_2 -adrenoreceptor agonists were proposed for the treatment of COPD (Dougall et al. 2003; Ind et al. 2003).

Lessons from transgenic mice for the relationship between asthma and COPD were discussed by Elias (2004).

Lappalainen et al. (2005) found that interleukin-1 β causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung.

Romano (2005) discussed selectin antagonists for their therapeutic potential against asthma and COPD.

Jones et al. (2002) described a model for the continuous monitoring of polymorphonuclear leukocyte trapping in the pulmonary vasculature of the rabbit.

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PROCEDURE

Guinea pigs of either sex weighing 300–400 g are used. The animal is placed in a cylindrical glass vessel, with 2 tubes at either ends. One serves as the entrance of the aerosol, the other for its efflux. The latter tube has a side-arm connecting to a tambour, from which changes in pressure can be registered. A pinch-clamp with a variable screw is placed on the efflux tube beyond the side arm, permitting the regulation of the sensitivity of the system, so that the normal respiration is not registered, while the displacement of air in the enclosure caused by coughing of the animal is registered. The guinea pig is exposed to the aerosol of 7.5% citric acid in water for 10 min. Each animal is tested first to obtain the control response. The number of tussive responses is registered. One hour later, the test substance is applied either s.c. or orally, and 30 min later the guinea pig is subjected to the aerosol again. The number of coughs during 10 min is recorded.

EVALUATION

The number of coughs after treatment is expressed as percentage of the control period. Using various doses, ED_{50} values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The citric acid induced coughing in guinea pigs has been proven to be an effective method to test antitussive agents.

MODIFICATIONS OF THE METHOD

The citric cough model in guinea pigs was used by Adcock et al. (1988) to study the effects of codeine, morphine and an opioid pentapeptide, by Hay et al. (2000) to study a potent and selective neurokinin-3 receptor antagonist, and by Brown et al. (2004) to study antitussive activity of sigma-1 receptor agonists.

Cough elicited by capsaicin inhalation in guinea pigs was used by Bolser et al. (1993, 1997) to study antitussive effects of GABA_B agonists or NK1 and NK2 tachykinin receptor antagonists, by McLeod et al. (1998) to study the antitussive action of antihistamines, and by Trevisani et al. (2004) to investigate the activity of iodo-resiniferatoxin, an ultra potent antagonist of the transient receptor potential vanilloid-1.

Forsberg et al. (1988) studied cough and bronchoconstriction mediated by aerosols of capsaicin or citric acid or nicotine or histamine in guinea pigs.

D.3 Antitussive Activity

D.3.0.1

Antitussive Activity After Irritant Inhalation in Guinea Pigs

PURPOSE AND RATIONALE

Cough is thought to be caused by a reflex. The sensitive receptors are located in the bronchial tree, particularly in the bifurcation of the trachea. These receptors can be stimulated mechanically or chemically, e. g., by inhalation of various irritants. Nerve impulses then activate the cough center in the brain. Several animals species and several irritants have been used, most frequently the citric acid induced cough in guinea pigs

Püschmann and Engelhorn (1978) studied the inhibition of the coughing reflex induced by inhalation of a citric acid spray in *rats*.

Other irritants have been used to induce cough, e. g. ammonia in dogs, guinea pigs, and cats (Rosiere et al. 1956; Källqvist and Melander 1957; Chen et al. 1960; Sallé and Brunaud 1960; Ellis et al. 1963), or nebulized sulfuric acid or sulfur dioxide in guinea pigs, rats, cats or dogs (Eichler and Smiatek 1940; May and Widdicombe 1954; Winter and Flakater 1952, 1954; Friebel et al. 1955; Reichle and Friebel 1955; Wiedemeijer et al. 1960; Chermat et al. 1966; Karttunen et al. 1982). Capsaicin aerosol was used by Forsberg and Karlsson (1986), Gallico et al. (1997).

Winter and Flakater (1955) exposed sensitized guinea pigs to aerosol of a specific antigen.

Kamei et al. (1989) induced cough in rats by a nebulized solution of capsaicin. The cough reflex was measured as airflow into or out of the chamber of a body plethysmograph by a pneumotachometer head.

Carotis sinus excitation in dogs induced by injection of lobeline resulting in coughing could be suppressed by codeine (Gross 1957).

Sanzari et al. (1968) induced cough in cats anesthetized with α -chloralose by intravenous injection of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a ganglionic stimulant which is more potent than lobeline and possesses only marginal ganglionic blocking properties. The number of coughs was found to be a linear function of the dose of the irritant. Coughs were recorded as spikes superimposed on the respiratory pattern. The method is suitable for quantitative evaluation of antitussive activity.

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D.3.0.2

Cough Induced by Mechanical Stimulation

PURPOSE AND RATIONALE

Cough can be induced by mechanical stimulation of the trachea in anesthetized guinea pigs (Takagi et al. 1960; Gallico et al. 1994).

PROCEDURE

Male guinea pigs weighing 350–400 g are maintained in conditioned quarters (temperature $21 \pm 2^\circ\text{C}$, relative humidity $55 \pm 10\%$, 12 h on-12 h off light cycle) with food and water ad libitum for at least 1 week before use.

After overnight fasting with water ad libitum, the guinea pigs are lightly anesthetized with 25% urethane (4 ml/kg i.p.) which induces surgical levels of analgesia without depressant effects on respiratory function. Analgesia is monitored throughout the experiment as the disappearance of head shaking in response to ear pinch. The animals are maintained at a constant body temperature of 37°C by means of a heated plate. A thin steel wire is gently inserted into the trachea through a small incision near the cricoid cartilage. Coughs are evoked by pushing the steel wire to reach the bifurca-

tion of the trachea 35 and 5 min before oral drug administration and 30, 60 and 120 min after treatment. One violent cough occurs upon each stimulation. Only those animals that respond to both mechanical stimulations before dosing are selected and then randomly assigned to receive the test drug at various doses or the standard (codeine 15, 30 and 60 mg/kg). Ten animals per dose are used.

EVALUATION

Evaluation of the statistical significance of the results is performed with Student's *t*-test for paired data. ED_{50} values are determined by logit transformation.

MODIFICATIONS OF THE METHOD

Several other ways of mechanical stimulation have been used, e. g., by a nylon-bristled stimulator thrust into the trachea in **dogs** (Kasé 1952, 1954), or by a silver thread in **decerebrated guinea pigs** (Lemeignan et al. 1966), or by vibration of an iron slung in the trachea of a dog induced by an electromagnet (Tedeschi et al. 1959) or electrical stimulation of the trachea via a bronchoscope (Gross et al. (1958) or through implanted copper electrodes (Stefko and Benson 1953; Benson et al. 1953; Granier-Doyeux et al. 1959; Stefko et al. 1961).

Hara and Yanaura (1959), Yanaura et al. (1974, 1982) induced cough in unrestrained animals after implantation of electrodes in the trachea.

Combined mechanical and chemical stimulation has been applied by Kroepfli (1950).

Bolser et al. (1999, 2001) and McLeod et al. (2002) studied the influence of antitussive drugs on the cough motor pattern in anesthetized cats. Coughing was produced by mechanical stimulation of the intrathoracic trachea with a thin flexible polyethylene cannula for 10 s per stimulus trial. During each trial, the cannula was repetitively moved in the trachea at a frequency of ~ 2 Hz. EMGs from the diaphragm and rectus abdominis muscles were recorded with the use of bipolar tungsten-wire electrodes. The diaphragm electrodes were placed through a small midline abdominal incision, which was subsequently closed. Cough is characterized by coordinated bursts of activity in inspiratory and expiratory muscles. Cough was defined as a large burst of EMG activity in the diaphragm that is immediately followed by a burst of EMG activity in the rectus abdominis muscle. This definition differentiates augmented breaths, the aspiration reflex, or the expiration reflex from cough. The antitussive activity of selected drugs was evaluated from cumulative dose responses after intravertebral artery administration of

each compound. The protocol consisted of the application of five consecutive mechanical stimulus trials after vehicle administration. Stimulus trials were applied at 1-min intervals after each dose of compound, for a total of five stimulus trials between doses. Approximately 7 min elapsed between each dose of compound.

Kasé et al. (1976) studied the antitussive activity of d-3-methyl-N-methylmorphinan in conscious mongrel dogs. Coughing was induced by mechanical stimulation with a stimulator consisting of 5 hog bristles on the mucosa of tracheal bifurcation through a chronically-built tracheal fistula and in lightly anesthetized cats with a stimulator consisting of 5 whiskers of a rabbit.

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D.3.0.3

Cough Induced by Stimulation of the Nervus Laryngicus Superior

PURPOSE AND RATIONALE

The probable pathways in the cough reflex arc are receptors in the area of the trachea and the large bronchi, afferent nerves mainly in the branches of the vagus nerve, a “cough center” located in the medulla oblongata, and efferent nerves closing the glottis and reinforcing the expiratory thrust. Stimulation of the Nervus laryngicus superior induces coughing. Antitussive agents with predominantly central action suppress the coughing reflex.

PROCEDURE

Cats of either sex weighing 2–3 kg are anesthetized with 40 mg/kg i.p. pentobarbital, placed on a heated operating table and their extremities secured. Since deep anesthesia suppresses coughing the dose of pentobarbital has to be adjusted. The fur is shaved ventrally at the neck. Small incisions are made at both sides of the larynx. The superior laryngeal nerves (forming a loop) are prepared carefully. After a median skin incision, the trachea is exposed and cannulated. The cannula is connected with a Fleisch-tube (size 00). One femoral artery is cannulated for registration of blood pressure via a Statham pressure transducer. One femoral vein is cannulated for intravenous application of test substances. Small hook electrodes are attached to each laryngeal nerve. At the end of an inspiration square wave impulses with a frequency of 50 Hz, an impulse width of 0.5 ms, an amplitude of 0.2–1.0 Volt, and a duration of 1–10 s are applied every 5 min. The intensity of the forced expiration is measured by the Fleisch pneumotachograph and recorded simultaneously with blood pressure on a polygraph. Prior to the intravenous application of the test compound, the response to three stimuli is recorded serving as control. After injection of the test compound or the

standard the stimuli are repeated every 5 min. Suppression or diminution of the forced expiration is recorded over 1 h. Then, the next dose or the standard (codeine phosphate 1–2 mg/kg i.v.) is applied.

EVALUATION

Total or partial suppression of the forced expiration are recorded over time and expressed as percentage of control. Intensity and duration of the effect are compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

The method described by Domenjoz (1952) is very useful to detect centrally active antitussive agents like codeine, but by definition can not determine compounds which act on cough receptors in the bronchial area. Moreover, even light anesthesia influences the cough reflex.

MODIFICATIONS OF THE METHOD

Several other assays have been described which elicit the cough reflex by central or nerve stimulation. Toner and Macko (1952) also stimulated the superior laryngeal nerve in anesthetized cats to induce a definite cough as indicated by rapid contractions of the abdominal musculature.

Mattalana and Borison (1955), Chakravarty et al. (1956) used decerebrated cats to study the central effects of antitussive drugs on cough and respiration. Cough responses were obtained by electrical stimulation of the dorsolateral region of the medulla with bipolar needle electrodes oriented by means of a stereotactic instrument.

Lindner and Stein (1959) evaluated derivatives of diphenyl-piperidono-propan, a series of antitussive drugs using a modification of the method originally described by Domenjoz (1952).

Schröder (1951) and Bobb and Ellis (1951) elicited cough in **conscious dogs** by stimulation of the vagus nerve in a surgically prepared skin loop. In anesthetized cats, coughs were elicited by electrical stimulation of the dorsolateral region in the upper medulla (Kasé et al. 1970).

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D.4

Effects on Tracheal Cells and Bronchial Mucus Secretion and Transport

D.4.0.1

In Vitro Studies of Mucus Secretion

PURPOSE AND RATIONALE

Mucus secretion has been studied in isolated tracheas from ferrets and dogs (Borison et al. 1980; Kyle et al. 1987).

PROCEDURE

Ferrets of either sex weighing 0.6 to 1.5 kg are anesthetized with sodium barbital intraperitoneally. The trachea is exposed and cannulated with a special Perspex cannula about 5 mm below the larynx. The animal is then sacrificed with an overdose of the anesthetic and the chest is opened along the midline. The trachea is exposed to the carina, cleared of adjacent tissue, removed from the animal and cannulated just above the carina. The trachea, with its laryngeal end down, is then mounted in a water-jacketed organ bath and bathed on its submucosal site with Krebs-Henseleit solution plus 0.1% glucose at 37°C and bubbled with 95% O₂ and 5% CO₂. The lumen of the trachea remains air-filled. A plastic catheter is inserted into the lower cannula to form an airtight seal into which secretions can periodically be withdrawn and collected. Volumes of secretions are estimated by the weight difference of catheter lengths with and without secretions.

Simultaneous measurements of both mucus secretion and changes in tissue volume *in vitro* are achieved by mounting portions of ferret trachea cut longitudinally along the posterior wall, flattened out and pinned to a Perspex chamber. Krebs-Henseleit solution at

37°C and gassed with 95% O₂ and 5% CO₂, is circulated on the submucosal side of the tissue, while the luminal side is exposed to the atmosphere. The surface area of the exposed tissue is about 50 mm². Mucus secretion is promoted by electrical field stimulation at 50–100 V, 20 Hz, 1–2 ms duration, applied through the pins holding the tissue. Before the start of each experiment, surface fluid is gently wiped off from the luminal surface with a tissue pledget. The epithelium is coated with a layer of powdered tantalum dust; as mucus secretion from submucosal glands occurs through gland ducts, the layer of tantalum effectively traps the secreted mucus above the duct and under the tantalum layer. Nearly hemispherical hillocks are formed. The surface is photographed at intervals through a dissecting microscope and hillock diameters are measured. Assuming the hillocks to be hemispheres, the secretion volume per unit area is calculated. Drugs are added to the submucosal bath.

EVALUATION

Secretory response after electrical stimulation in the presence or absence of drugs is recorded after 45, 90, and 135 min.

MODIFICATIONS OF THE METHOD

Quinton (1979) used isolated tracheae from cats. A segment of the trachea was mounted in a chamber such that the serosal side was constantly bathed in Ringer solution, whereas the epithelial surface was coated with water-saturated paraffin oil. Secretion was stimulated by adding appropriate drug concentrations to the bath. Under a dissecting microscope, small droplets of secretory fluid were observed to form on the tracheal epithelial surface shortly after stimulation. Timed collection of droplets secreted from three to four glands were taken up between oil blocks in constant-bore capillaries (78 µm inner diameter), and droplet volumes were measured for rate determinations usually over a period of 5 min.

CRITICAL ASSESSMENT OF THE METHODS

Both modifications of the *in vitro* methods need at least as many animals and are as time consuming as the *in vivo* methods.

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D.4.0.2

Acute Studies of Mucus Secretion

PURPOSE AND RATIONALE

Many diseases of the respiratory tract cause both qualitative and quantitative changes in the mucus that covers and protects the airway epithelium. To study the influence of drugs, methods of collecting bronchial mucus are necessary (Braga 1988). Perry and Boyd (1941) described a method for collecting bronchial mucus from the rabbit.

PROCEDURE

Rabbits weighing 2.5 to 3.5 kg are anesthetized by intraperitoneal injection of 1.1 to 1.4 g/kg urethane. The trachea is exposed by blunt dissection and half opened, 2 cm below the cricoid cartilage. One arm of a T cannula with a large enough diameter to slightly distend the trachea is inserted into the trachea. The perpendicular arm is connected to an air outlet of a humidifier (temperature 35–38°C, relative humidity 80%). The other arm is connected to a collection tube. The rabbit is restrained in the supine position on a 60-degree inclined board with his head downward. Respiratory tract fluids are collected in centrifuge tubes at one hour intervals. Mucus secretion can be stimulated by vagal stimulation or by ammonium chloride given by stomach tube or by pilocarpine given i.p.

EVALUATION

Time response curves after stimulants of mucus secretion are compared with data from untreated animals.

MODIFICATIONS OF THE METHOD

A method for collecting mucus from cats, using a segment of cervical trachea about 5 cm long isolated *in situ*, with nerve and blood supplies intact and a glass cannula inserted to each end, has been described by Gallagher et al. (1975).

A method to collect mucus from the upper tract trachea and the nasopharynx in dogs in acute experiments has been proposed by Proctor et al. (1973).

Engler and Szelenyi (1984) described a new method for screening mucosecretolytic compounds using tracheal phenol red secretion in **mice**. Phenol red at a dose of 500 mg/kg was injected intraperitoneally to male mice. Thirty min later, the animals were sacrificed by carbon dioxide. The whole trachea was dissected free from surrounding tissue and excised. Each trachea was washed for 30 min in 1 ml physiological saline. Afterwards, 0.1 ml 1 M NaOH was added to the washing to stabilize the pH of the lavage fluid. The concentration of phenol red was measured photometrically. Agonists were administered subcutaneously 15 min or intragastrally 30 min before phenol red was injected. Antagonists were given 5 min prior to the administration of agonists.

Other dyes, such as Evans blue or sodium fluorescein also are reported to be eliminated in the respiratory tract fluid of mice (Graziani and Cazzulani 1981).

Dye methods reported for mice can also be used for rats (Quevauviller and Vu-Ngoc-Huyen 1966). Alcian blue was used to stain the normal bronchial tree. After chronic treatment with sulfur dioxide, there were changes in bronchial coloration. Administration of drugs protected against the effects of sulfur dioxide.

Secretion from tracheal submucosal glands can be studied in **dogs** (Davis et al. 1982; Johnson and McNee 1983, 1985). In anesthetized dogs the epithelial surface of the upper trachea is exposed and coated with powdered tantalum. Secretions from the submucosal gland ducts form elevations (hillocks) in the tantalum layer. The number of hillocks that appear in a 1.2 cm² field is counted.

A micropipette method for obtaining secretions from single submucosal gland ducts *in vivo* in **cat** tracheas has been described (Ueki et al. 1979, 1980; Leikauf et al. 1984). In anesthetized cats an endotracheal tube was inserted into the lower trachea and connected to a constant volume respirator. The remainder of the trachea above the endotracheal tube was then dissected open by a midline incision. Paraffin oil equilibrated with HEPES buffer was then placed on the exposed mucosa to prevent drying and to aid visualization of the gland duct openings. The secretions from the gland duct openings were collected with constant-bore (99 µm ID) glass micropipettes. The volume and the viscosity of the secreted mucus were determined.

CRITICAL ASSESSMENT OF THE METHODS

For the methods of mucus collection in rabbits, cats or dogs a rather high number of animals is necessary to achieve data suitable for statistical analysis. For screening procedures the methods using dye elimina-

tion into the trachea of mice or rats seem to be preferable.

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D.4.0.3

Studies of Mucus Secretion With Chronic Cannulation

PURPOSE AND RATIONALE

Several techniques have been developed for chronic collection of mucus (Wardell et al. 1970; Yankell et al. 1970; Scuri et al. 1980).

PROCEDURE

Beagle dogs weighing 9–11 kg are anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium. The cervical trachea is exposed by a midline skin incision and blunt dissection of the muscles. A segment approximately 10 rings in length,

with an intact blood and nerve supply, is transected. The cephalic and caudal parts of the trachea are anastomosed end-to-end with interrupted gut sutures to reestablish a patent airway. The isolated segment is loosened slightly from the surrounding tissue and turned 180° to reverse cilia movement. A funnel-shaped silicone cannula is attached to the outer surface of the proximal end of the tracheal segment with surgical mesh and sutured in place. With cannulation completed, the tracheal segment is placed in a pocket below the sternohyoid muscle and the cannula brought to the surface and exteriorized through a stab wound. Alternatively, the isolated segment is closed at its caudal end with interrupted gut sutures. The mucosal surface of the cervical end of the isolated tracheal segment is sutured with interrupted silk sutures to the overlying subcutaneous tissue through a small incision in the cervical skin. Muscles and skin are sutured normally. In two or three weeks the skin heals over the small stoma resulting in a subcutaneous pouch of functioning tracheal tissue. Mucus samples can be collected for months. In this modification, a balloon can be placed into the pouch. Pressure changes in the balloon due to contraction of the smooth tracheal muscles after physostigmine injection or vagal stimulation or relaxation after atropine injection are recorded demonstrating parasympathetic innervation.

EVALUATION

Parasympathomimetic stimulation (0.5 mg/kg pilocarpine s.c.) increases the flow rate of tracheal fluids. Pressure changes in the balloon after injection of parasympathomimetic or sympathomimetic drugs are compared with baseline values.

MODIFICATIONS OF THE METHOD

Scuri et al. (1980) inserted a T-shaped cannula into the trachea of anesthetized rabbits. The wound was sutured, the third arm was connected with a collecting tube, and after 3 days of antibiotic administration, the mucus was collected at different times to establish basal production. For the experiments, mucus was collected during a 4 h control period, then drugs were given intravenously, orally or as aerosol inhalation. Mucus was further collected during the periods of 0 to 4 and 4 to 24 h and analyzed for sialic acid, fucose, and protein content.

A tracheal pouch method in ferrets has been described by Barber and Small (1974).

Several authors published methods to determine **viscoelastic properties and rheological behavior of tracheal and bronchial mucus**: Philippoff et al.

(1970), Lopez-Vidriero and Das (1977), Martin et al. (1980) Kim et al. (1982), Braga (1988), King (1988), Majima et al. (1990).

CRITICAL ASSESSMENT OF THE METHODS

The methods using pouches in dogs may be useful for physiological studies, but for pharmacological purposes the rabbit method of Scuri et al. (1980) seems preferable.

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D.4.0.4

Bronchoalveolar Lavage

PURPOSE AND RATIONALE

Isolation of bronchial cells from bronchoalveolar lavage was described by Myrvik et al. (1961), Bassett et al. (1988), Fryer et al. (1994, 1997), Wang et al. (1997).

PROCEDURE

After determination of mechanical respiratory parameters in anaesthetized guinea pigs, bronchoalveolar lavage is performed via the tracheal cannula. The lungs are lavaged with 5 aliquots of 10 ml phosphate-buffered saline containing 3 mM EDTA and 100 μ M isoproterenol (pH 7.2–7.4). The recovered lavage fluid (40–45 ml) is centrifuged, the cells are resuspended in 20 ml of phosphate-buffered saline, and total cells are counted using a hemacytometer. The remaining aliquot is centrifuged again and cells are stained to determine cell differentials.

EVALUATION

The differences in cells recovered from bronchoalveolar lavage between treatment groups are tested by use of a one-factor analysis of variance. $P < 0.05$ is considered significant.

MODIFICATIONS OF THE METHOD

Gossart et al. (1996) determined TNF- α activity in the supernatant of bronchoalveolar lavage by the cytotoxicity against TNF- α -sensitive L929 murine fibroblasts.

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of secreted mucus. Many attempts have been made to visualize and to quantify this phenomenon. Several authors used the light beam reflex method which can be used for *in vivo* as well as for *in vitro* experiments (Dalhamn 1956, 1964; Dalhamn and Rylander 1962; Hakansson and Toremalm 1963; Mercke et al. 1974; Baldetorp et al. 1976; Lopez-Vidriero et al. 1985).

PROCEDURE

Rats are anesthetized by intraperitoneal injection of tribromo-ethanol (Avertin). The trachea is exposed and its soft parts are incised by electrocoagulation so that bleeding is avoided. The cartilaginous rings are opened sufficiently to permit microscopy. The rat is immediately placed in a moist chamber. The trachea opening is linked to a microscope (Leitz Ultropak) by means of a rubber bellows which is fitted around the lens of the microscope and is made to embrace the trachea by means of a piece of rubber tubing that is slit along its length and secured to the bellows. The beam of an illuminating lamp is concentrated to a surface of about one mm². By placing a heat-reflecting filter in the path of the beam the rise in temperature can be reduced. For registration of the reflected light high speed cameras with a speed of 220 exposures are used. Alternatively, the reflected light from the microscope is directed to a TV camera and amplified to be displayed on a TV screen. The frequency of ciliar beats is recorded over one hour.

EVALUATION

The beat frequency of treated animals is compared with that of controls.

MODIFICATIONS OF THE METHOD

Mercke et al. (1974) described a stroboscopic method for standardized studies of mucociliary activity in rabbit tracheal mucosa.

Lierle and Moore (1935) inserted windows into anesthetized rabbits for observation of the ciliary activity in the maxillary sinus of living animals.

With modern equipment, a similar technique has been used by Hybbinette and Mercke (1982a–c), Lindberg and Mercke (1986), Lindberg et al. (1986), Mercke et al. (1987) to study the role of several pharmacologic agents in the mucociliary defense of the rabbit maxillary sinus.

Corssen and Allen (1958) compared the toxic effects of various local anesthetic drugs on human ciliated epithelium *in vitro* by observation of rotating globes of human tracheal epithelium in tissue culture.

D.4.0.5**Ciliary Activity****PURPOSE AND RATIONALE**

Ciliary activity is a natural defense mechanism of the mucosa in the respiratory tract against harmful extraneous agents resulting in a continuous transportation

Cheung (1976) performed high speed cinemicrographic studies on rabbit tracheal (ciliated) epithelia.

Iravani (1967, 1971, 1975) studied the ciliary activity in the intrapulmonary airways of rats by incident light microscopy.

Lee and Verdugo (1976), Verdugo et al. (1980) recommended laser light-scattering spectroscopy for the study of ciliary activity.

Manawadu et al. (1978) studied the effects of local anesthetics on ciliary activity using ferret tracheal rings *in vitro*. The ferret possesses a long neck, and 100 or more tracheal rings can be obtained from a single ferret. The tracheal rings were maintained in sterile tubes. The ciliary activity was graded by determining the percentage of cilia beating on each ring, using transmitted light and the 10× objective of an inverted microscope.

Rutland and Cole (1980) and Hesse et al. (1981) used a non-invasive method for obtaining nasal ciliated epithelium which is suitable for measurement of ciliary beat frequency.

Van de Donk et al. (1980) used isolated chicken embryo tracheas to measure the effects of preservatives on ciliary beat frequency. Maurer et al. (1982) studied the role of ciliary motility in acute allergic mucociliary dysfunction in cultivated ciliated cells from sheep.

Lopez-Vidriero et al. (1985) studied the effect of isoprenaline on the ciliary activity of an *in vitro* preparation of rat trachea.

Braga et al. (1986) described a simple and precise method for counting ciliary beats directly from the TV monitor screen using specimens of human ciliated epithelium obtained by brushing the nasal mucosa.

Curtis and Carson (1992) used pieces of human nasal epithelium for computer-assisted video measurement of ciliary beat frequency *in vitro*. Ciliary beat frequency was viewed with a microscope equipped with a phase contrast objective. The microscopic image was recorded by a camera and data stored by a video-recorder. For measuring ciliary beating, tapes were displayed with amplification on a monitor. A photoelectric transducer was positioned over the video image of the cilia. Movement of the cilia interrupting the light path caused changes in light intensity recorded by the photocell transducer.

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D.4.0.6

Studies of Mucociliary Transport

PURPOSE AND RATIONALE

Mucus flow has been studied in *in vitro* and *in vivo* experiments (Irvani 1971; Ahmed et al. 1979). The rate of mucus flow can be estimated by measuring the time needed for certain particles to travel a known distance in the trachea. Numerous substances have been used such as charcoal particles (Dalhamn 1956), Teflon discs (Ahmed et al. 1979), and other pulverized materials (Deitmer 1989). The effect of local radioactivity on tracheal mucous velocity of sheep has been studied *in vitro* and *in vivo* by Ahmed et al. (1979).

PROCEDURE

For *in vitro* experiments, sheep are sacrificed during anesthesia, the trachea is exposed, clamped, and resected just below the cricoid cartilage. The chest is opened and the trachea is resected at the level of the carina. Then the trachea is slit open along the posterior membranous wall, pinned with gentle stretching on a board, and slanted upward at an angle of 25°. A metric ruler is placed along the board as a measuring reference. The board is then placed in a Plexiglas chamber with a constant temperature of 37°C and 100% humidity. Teflon discs are spread on the mucous layer. Tracheal mucus velocity is estimated by filming the movements of the radioopaque Teflon discs visualized on a television monitor connected to a camera. Disc motion is recorded for 1–2 min on a videotape, and the distance measured during the elapsed time is obtained from the videomonitor.

For *in vivo* measurements of tracheal mucus velocity, the roentgenographic method of Friedman et al.

(1977), Sackner et al. (1977) is used. The sheep are restrained, and their heads are immobilized with a sling. The nasal mucosa is sprayed with a 2% lidocaine solution for topical anesthesia. A bronchofiberscope is inserted transnasally, and its tip is placed just below the vocal cords. Radioopaque Teflon discs 1.0 mm in diameter, 0.8 mm thick, and weighing 1.76 mg are blown through the inner channel of the bronchofiberscope onto the tracheal mucosa in a circumferential distribution. The cervical trachea containing the discs is visualized in the lateral projection with a television monitor. Disc motion is recorded on a videotape while the time is displayed on a digital clock. The disc image is marked to obtain the distance traveled. This distance is measured with a ruler, and the linear velocity of the disc is computed by dividing the distance by the elapsed time. This procedure is repeated for all discs in the field of view. To compute a mean tracheal mucus velocity, data from 10–15 discs are obtained in each filmed run.

EVALUATION

Disc velocities measured *in vitro* or *in vivo* are compared before and after treatment.

MODIFICATIONS OF THE METHOD

Mucociliary transport has been studied on the hard palate of decapitated frogs measuring the transport velocity of small particles, e. g., pieces of cork or charcoal (Kochmann 1930; Sadé et al. 1970).

Suzuki (1966) measured the movement of a standard object (1 mm² aluminum foil) on the ciliated surface of the palate of frogs.

Mucociliary clearance by the *in vitro* frog method was used by Leitch et al. (1985) to study the effects of ethanol.

Movement of poppy seeds in rabbit tracheal preparations was studied by Kensler and Battista (1966).

In chicken nasal mucosa the interaction between mucociliary transport and the ciliary beat was studied by Ukai et al. (1985).

Mucus transport in the respiratory tract of anesthetized cats was measured with uniform particles of lycopodium spores triturated with lamp black (Carson et al. 1966).

Mucociliary clearance velocities were determined by a radioisotopic method in dogs (Giordano et al. 1977, 1978).

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D.4.0.7

Culture of Tracheal Epithelial Cells

PURPOSE AND RATIONALE

Marked morphological changes of the airway epithelium, up to severe damage, are frequently observed in inflammatory airway diseases and appear to play an important role in the pathogenesis of the broncho-obstructive symptoms (Webber and Corfield 1993; Hay et al. 1994). Freitag et al. (1996) studied the effects of lipopolysaccharides (LPS) and TNF- α on cultured rat tracheal epithelial cells and determined NO synthase activity.

PROCEDURE

Trachea of newborn rats, cut into small pieces, are explanted with the epithelial surface downwards onto 60 mm culture dishes (Lechner et al. 1985) and cultured in low-calcium (60–80 μ mol/l) RPMI-1640 medium at 37°C and 5% CO₂. The medium containing 16% fetal calf serum is supplemented with epidermal growth factor and other growth promoting factors, such as 80 ng/ml cholera toxin, 2.6 ng/ml estradiol, 180 ng/ml hydrocortisone, 2.5 μ g/ml insulin, 12.5 μ g/ml transferrin, and 100 U/ml penicillin, 100 μ g/ml streptomycin and 3.5 μ g/ml amphotericin B to allow a selective outgrowth of epithelial cells (Emura et al. 1990). Confluent epithelial layers are obtained after about 6–10 weeks at which time all cells show a positive staining with a pancytokeratin-antibody. Confluent cells of following passages are treated for four subsequent days with LPS (10 μ g/ml) or TNF- α (500 U/ml).

The **morphology** of the cells is evaluated is evaluated by daily inspection using a phase contrast microscope with photographic documentation. Cell density in the culture is determined in each culture dish by counting daily the number of cells in four marked areas (each 2000 μ m²). For immunocytochemical staining, the cells are fixed by incubation in 100% methanol at –20°C for 20 min. The cells are washed with Ca/Mg-free phosphate buffered saline and several areas are marked of by nail-varnish. A solution of the anti-cytokeratin(pan)-antibody (monoclonal mouse IgG, Boehringer Mannheim) is added, incubated for 2 h at room temperature and after washing incubated for 30 min with a secondary antibody (FITC coupled, polyclonal rabbit anti-mouse IgG, Sigma). After washing, fluorescence microscopy is performed using a microscope with a FITC-specific filter combination. Unspecific fluorescence is excluded by performing the staining procedure in a different area of the same culture, but without the addition of the primary antibody.

For **determination of NO synthase activity**, confluent cultures are washed with oxygenated and prewarmed (37°C) Krebs-HEPES medium. Then the cells are incubated for 1 h in the same medium containing 37 kBq ³H-L-arginine (100 nmol/l). After collection of the supernatants the cells are extracted in 1 ml of 0.4 mol/l HClO₄ for 2 h at 0–4°C.

By **HPLC analysis** ³H-compounds (³H-L-citrulline, ³H-L-ornithine and ³H-L-arginine) in incubation media and cell extracts are separated on a reverse phase column (length 250 mm, inner diameter 4.6 mm, pre-packed with Shadon ODS-Hypersil, 5 mm) using as mobile phase 0.1 mmol/l sodium phosphate buffer

(adjusted to pH 1.8) which contains octane sulphonic acid sodium salt (400 mg/l), Na₂EDTA (0.3 mmol/l) and methanol (6.25% v/v) with a flow rate of 1 ml/min (Hey et al. 1995). The eluate is collected in 1 min fractions into counting vials. After addition of a commercial scintillation cocktail the radioactivity is determined by liquid scintillation spectrophotometry. External standardization is used to correct for counting efficiency. The retention time is determined by the use of ¹⁴C-labelled (L-citrulline or L-ornithine) or ³H-labelled (L-arginine) standards. Protein content in cells is determined by a commercially available assay.

EVALUATION

The amounts of ³H-L-citrulline in supernatants or cell extracts are expressed as DPM/μg protein. Changes in cell density are expressed as % of the density observed in each culture dish at the start of the experiment. Mean values are given ± SEM. The significance of difference is evaluated by Student's *t*-test.

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D.4.0.8

Alveolar Macrophages

PURPOSE AND RATIONALE

Alveolar macrophages have been used for various purposes. A rat pulmonary alveolar macrophage cell line (NR8383) was initiated in culture by Helmke et al. (1987) in the presence of a gerbil lung conditioned medium and has been propagated continuously in culture. Sun et al. (1999) tested the inhibition

of Ca²⁺ influx by pentoxifylline in NR8383 alveolar macrophages.

PROCEDURE

Cell Culture

The NR8383 alveolar macrophages cell line is grown in plastic tissue culture flasks in Ham's F12 medium containing 15% fetal bovine serum, 100 g/ml penicillin and 100 U/ml streptomycin sulfate. The alveolar macrophages are cultured at 37°C in an atmosphere of 5% CO₂ in air. The medium is routinely changed twice weekly. The viability of alveolar macrophages is routinely measured by trypan blue exclusion before and at 1.5 h after fura-2 loading, when all [Ca²⁺]_i determinations are completed.

Determination of [Ca²⁺]_i

[Ca²⁺]_i is determined using the Ca²⁺-sensitive fluorescent indicator fura-2 (Zhang et al. 1997; Mörk et al. 1998). NR8383 alveolar macrophages are loaded with fura-2 by incubation in PSS containing 2 mM (final concentration) fura-2/AM and 0.01% BSA for 20 min at 37°C. The cells are then rinsed twice with PSS containing 0.01% BSA and resuspended in the same medium (1.75 × 10⁶ cells/ml). For [Ca²⁺]_i determination, a 2-ml aliquot of fura-2-loaded cells is centrifuged at 50 g for 2 min, resuspended in PSS containing 0.01% BSA and placed in a 4-ml cuvette. [Ca²⁺]_i is measured at 37°C using a PTI Deltascan fluorometer (PTI, South Brunswick, N.J., USA). The excitation wavelengths used are 340 and 380 nm and the emission wavelength is 505 nm. Calibration of [Ca²⁺]_i is performed for each measurement trace. Then, 1 mM CaCl₂ (for Ca²⁺-free medium) and 50 mM ionomycin are added to obtain the limiting ratio for the Ca²⁺-saturated form (*R*_{max}) of fura-2. Then, 0.0005% digitonin and 10 mM EGTA are added to obtain the limiting ratio for the free form of fura-2 (*R*_{min}). Fluorescence ratios of the 340- and 380-nm excitation and 505-nm emission are converted to [Ca²⁺]_i according to Grynkiewicz et al. (1985) using 224 nM as the *K*_d of fura-2 for Ca²⁺.

Measurement of Store-Depletion-Activated Ca²⁺ Influx

The Ca²⁺ influx activated by depletion of the inositol 1,4,5-trisphosphate- (IP₃-) sensitive intracellular store is measured according to Zhang et al. (1997) and Zhu et al. (1998). Cells are exposed to either ATP or thapsigargin (TG) in Ca²⁺-free medium for 5 min, and 1 mM Ca²⁺ is then added to initiate Ca²⁺ influx. The initial linear portion of [Ca²⁺]_i changes after addition of Ca²⁺ is used to calculate Ca²⁺ influx rate (nM/min).

EVALUATION

Results are presented as the mean \pm SEM of separate determinations using different cell preparations. Comparisons are made using the unpaired Student's *t*-test or the analysis of variance. *P* values <0.05 are considered significant.

MODIFICATIONS OF THE METHOD

Sirois et al. (2000) studied the influence of histamine in the cytokine network in the lung through H₂ and H₃ receptors. Alveolar macrophages from humans, Sprague Dawley rats and the alveolar macrophage cell line NR8383 were treated with different concentrations of histamine prior to their stimulation with suboptimal concentrations of lipopolysaccharide (LPS). Release of tumor necrosis factor (TNF) and interleukin-10 (IL-10) was measured.

Using the rat alveolar macrophage cell line NR8383, Gazin et al. (2004) found that uranium induces TNF- α secretion and activates the p38 mitogen-activated protein kinase (p38 MAPK).

Yang et al. (2004) studied in mice the synergy between a signal transducer and activator of transcription 3 and retinoic acid receptor- α in the regulation of the surfactant protein B gene in the lung.

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D.5 Safety Pharmacology of the Respiratory System

See Murphy (2006).

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Chapter E

Psychotropic and Neurotropic Activity

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E.1 **Effects on Behavior and Muscle Coordination**

E.1.1 **Spontaneous Behavior**

E.1.1.1 **General Considerations**

The effects of drugs on the central and peripheral nervous systems can be easily recognized in normal animals. This does not necessarily mean that these effects can be used in therapy. Observing the global effects of drugs during LD₅₀-determinations, pharmacologists can detect psychotropic activity. Only, if these effects occur also in doses considerably below the LD₅₀, are further evaluations justified. This basic experience resulted in the development of a variety of observational tests and activity measurements.

E.1.1.2 **Observational Assessment**

PURPOSE AND RATIONALE

A systematic, quantitative procedure assessing the behavioral state of mice for the evaluation of drugs has been described by Irwin (1964, 1968). The method is applied in the beginning of pharmacological screening to detect psychotropic activities. It allows to identify and differentiate the profile pattern of various classes of pharmacological agents. Furthermore, observational assessment allows into the safety and potential toxicity profile of a new drug.

PROCEDURE

Mice of either sex (NMRI-strain) with a weight between 18 and 22 g are kept under standard laboratory conditions 5 days before the experiment. Animals tested for oral administration have water ad libitum but are deprived of food 16 h before the test. Animals for intravenous or intraperitoneal administration have access to food and water ad libitum until the test. For unknown substances the following doses are given:

- i.v. administration: 100, 200, 400 mg/kg
- i.p. administration: 300, 600, 1200 mg/kg
- oral administration: 500, 1000, 2000 mg/kg

Three animals are used for each dose. One group of 3 animals receiving the vehicle only, serves as control group.

Immediately after drug administration, the animals are closely observed for 2 h (following i.v. or i.p. administration) and for 5 h (following oral administra-

tion) by a “blind” observer. The following parameters are checked and compared to the vehicle control group:

| Effects on CNS | Effects after manipulations |
|---------------------------------|------------------------------------------------|
| spontaneous motor activity | auditory stimulus response |
| restlessness | escape after touch |
| grooming behavior | righting reflex |
| squatting | paresis of hind limbs |
| staggering | paresis of forepaws |
| ataxic gait | cataplexy in induced positions |
| lying flat on the belly | Effects on reflexes |
| lying flat on the side | |
| lying flat on the back | pinna reflex |
| sleeping | corneal reflex |
| narcosis | pain following stimulation |
| bizarre behavior | Effects on autonomic nervous system |
| timidity | |
| Straubs’s phenomenon | pupil diameter (constriction or dilatation) |
| writing | |
| tremors | eyelids (closure or exophthalmus) |
| twitches | secretion of sweat |
| opisthotonus | salivations |
| clonic convulsions | lacrimation |
| tonic convulsions | cyanosis |
| rolling and jumping convulsions | piloerection |
| | defecation |
| | urination |

Depending on the route of administration, the observations are performed at different time intervals: after intravenous and after intraperitoneal administration during the first 30 min, and after 1 and 2 h; after oral administration during the first 60 min, and after 2 and 6 h. The number of deaths is counted during the first 24 h and after 7 days in order to evaluate acute and late toxicity. Arbitrary scores are chosen for each symptom. If positive effects are seen with the lowest dose, the experiment is repeated with lower doses being decreased by a factor of 3.

CRITICAL ASSESSMENT OF THE TEST

The test has been used by almost every laboratory in the world involved in screening of potential new drugs. Almost every laboratory has introduced its own modifications. In particular, the scores and the calculations differ from one laboratory to the other. Additional tests, such as grip strength or rotarod, described below,

have been included into the primary screen. Graph-bar profiles have been established for known drugs in order to rate new substances accordingly. The test is definitively an useful tool for primary screening resulting in hints on psychotropic activity but also for actions on other systems. Nevertheless, this test can not substitute for more sophisticated tests for the evaluation of psychotropic activity.

MODIFICATIONS OF THE METHOD

Based on the guidelines of the United States Environmental Protection Agency (USEPA) (1991) Mattsson et al. (1996) described a performance standard for clinical and functional observational battery (FOB) for examinations of **rats**. The performance standard was an idealized composite of FOB data from experienced laboratory personnel, each person tested on a separate set of four groups of rats. The rats were examined in random order and treatments were either (a) saline, (b) chlorpromazine, (c) atropine, followed by physostigmine or (d) amphetamine.

Testing of neurotoxicity with new pharmaceuticals was reported by Haggerty (1991). He proposed a primary screen for rodents, consisting of a functional observation battery and an automated test for motor activity. In addition, a functional observation battery for dogs was developed. The rat tier I screen assesses such functions as home cage and open field activity, stimulus reactivity, and neuromuscular function. The dog tier I screen emphasizes evaluation of gait, postural reactions, and reflex function.

Rambert (2000) underlined the importance of some general conditions for general pharmacology of the CNS, such as choice of the animal species, administration route and controls of the experimental context (surroundings, temperature, schedules of the trials, interference with food-intake episodes, influence of noises and smells, expertise of the experimental staff).

Crawley and Paylor (1997), Crawley (2000) proposed a test battery and behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Several neurological and neuropsychological tests are described which can be used as first screen for behavioral abnormalities in mutant mice. Multiple behavioral paradigms are included for several categories, such as neurological reflexes, sensory abilities, motor functions, learning and memory, feeding, sexual behavior, analgesia, aggression, anxiety, depression, schizophrenia, and drug abuse.

Detailed procedure of the Irwin test performed in rats is given by Porsolt (2006).

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E.1.1.3**Safety Pharmacology Core Battery****PURPOSE AND RATIONALE**

Regulatory agencies (EMA 1997, 2000) have pointed out the necessity to perform safety pharmacology studies before application of a new drug to human beings and to assess most carefully any potential side effects. Organs or systems acutely necessary for life, i. e., the cardiovascular, respiratory and central nervous systems, are considered to be the most important to assess in safety pharmacology studies. The following parameters of CNS activity should be assessed appropriately: motor activity, behavioral changes, coordination, sensory/motor reflexes and body temperature.

Detailed descriptions of tests are found for

Observation tests:

E.1.1.2 Observational assessment

Motor activity and behavior:

E.1.2.2 Method of intermittent observations

E.1.2.3 Open field test

E.1.2.4 Hole-board test

E.1.2.5 Combined open field test

Coordination:

E.1.3.1 Inclined plane

E.1.3.2 Chimney test

E.1.3.3 Grip strength

E.1.3.4 Rotarod method

Sensory/motor reflexes:

E.1.3.6 Influence on polysynaptic reflexes

E.1.3.7 Masticatory muscle reflexes

Follow-up studies for CNS safety pharmacology are recommended, such as behavioral pharmacology, learning and memory, specific ligand binding, neurochemistry, visual, auditory and/or electrophysiology examinations. Detailed descriptions of these tests are found in the respective chapters, e. g.,

E.1.2.6 EEG analysis from rat brain by telemetry.

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E.1.2**Effects on Motility****(Sedative or Stimulatory Activity)****E.1.2.1****General Considerations**

A survey on methods to evaluate depressants of the central nervous system has been published by Turner (1965). Many of these tests are still valid in spite of the fact that new classes of drugs have been introduced since that time which have not only augmented the therapeutic armamentarium but also changed the battery of pharmacological tests.

Sedative properties of drugs are tested mostly in mice or rats. Their spontaneous motor activity depends on various factors, such as the social situation (one or more animals), familiarity with the test environment, light and temperature. The term spontaneous motor activity includes different types of movements, such as locomotion, rearing, sniffing, grooming, eating and drinking. These phenomena can be well recognized by a skilled observer, but are difficult to record over long periods of time and to quantitate. Therefore, besides procedures based on observation many methods

for automatic registration have been developed. Almost every pharmacologist working in this field has designed his/her own apparatus. Several attempts have been made to measure not only simple locomotion, but also rearing and other types of movement. The conditions to characterize drug effects by measuring locomotor activity have been surveyed by Kinnard and Watzman (1966), Geyer (1990).

A special phenomenon, called "thigmotaxis" which means that rats have the tendency to remain close to the walls of the cage, has been described by Barnett (1963). Moreover, methods to measure curiosity have been recommended. Only a few examples of prototypic methods and equipment can be given.

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E.1.2.2

Method of Intermittent Observations

PURPOSE AND RATIONALE

The method described by Ther (1953) was designed to study stimulant and sedative drugs. The use of 3 mice per group implied a special social situation of the animals. For testing sedative activity, the mice are additionally treated with a stimulant, and for testing stimulant activity with a sedative drug. Each group of mice is observed repeatedly only for a short period of time during one hour and compared to a control group by a "blind" observer.

PROCEDURE

Mice of either sex with an average weight of 25 g are deprived of food and water for 24 h before the test. To avoid any influence of the circadian rhythm the experiment is performed only between 8:00 and 12:00 A.M. Twelve animals are divided into groups of 3 mice. One group of 3 mice serves as control group, the other groups receive different doses of the test drug intraperitoneally. For testing sedative activity, the mice are injected after 10 min subcutaneously with 0.5 mg/kg methamphetamine. For testing stim-

ulant activity, the mice are treated with 800 mg/kg paraldehyde. Ten minutes afterwards, each group is placed into a glass jar of 12 cm diameter and 20 cm height which in turn are placed in a wooden box of 130 × 50 × 30 cm. The glass jars are illuminated from above. Ten minutes after administration of methamphetamine or paraldehyde the observation is started.

EVALUATION

During 1 h the observer looks every minute for 1 s to each jar and registers if none, one, two or all three mice show any characteristic change in locomotion, rearing, grooming or sniffing. The maximum count in 1 h would be 180. Generally, activity decreases within 1 h. Nevertheless, methamphetamine treated animals have a total count between 120 and 150. Groups treated with an effective sedative drug show a dose dependent decrease of total counts. The number of counts in the treated groups is calculated as percentage of controls. From dose-response curves ED_{50} -values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method gives reliable results for sedative drugs and for compounds with central depressant activity, such as antihistaminics, neuroleptics and hypnotics. The disadvantage of the method lies in the fact that a skilled and trained observer is needed in order to get reproducible results. Therefore, several attempts have been made to automatize the method of intermittent observation.

MODIFICATIONS OF THE METHOD

Schaumann and Stoepel (1961) followed the principle of intermittent observation using a camera mounted above small wire cages (10 × 10 cm). The mice in the cages were photographed with an exposure time of 3 s every 7,5 min over a period of 2,5 h. Mice without movements give clear pictures, calculated as zero. Slight movements induce blurred contours, calculated as 1 point, and major movements give completely blurred pictures, calculated as 2 points. Normal mice show 20 or less points during 20 observations. Mice with more than 20 points are considered to be stimulated, mice with less than 5 points to be sedated. In this way ED_{50} -values can be calculated.

Vogel and Ther (1963) published an apparatus with automatic registration of intermittent observations. 18 cages are used which have a freely movable bottom with minimal weight which is supported by springs. A small permanent magnet attached below the floor induces an electrical current in a coil if the bottom is moved. Two mice are brought into each

cage. The control device registers during a variable period between 0.5 and 3 s if the bottom of one cage is moved or not. Within one minute, all 18 cages are registered successively. The number of movements within one hour is recorded for each cage. The sensitivity of the electric induction system is variable. A calibration is possible which picks up every movement of the animal, however, does not register the movements due to breathing. Dose-response curves can be established for stimulant and sedative drugs.

A similar apparatus was used by Hirabayashi and Tadokoro (1993) and Namina et al. (1999). The ambulatory activity of mice was measured by the tilting cage method using the Ambulometer, Model AMB-M1 (O'Hara, Tokyo, Japan).

Meyer (1962) measured the time until the exploration motility decreased to one third of the initial value.

Koek et al. (1987) used the principle of intermittent observations for various activities of rats such as locomotion, rearing, sniffing, licking, gnawing, grooming, loss of righting, Straub tail, ect. in order to compare the behavioral effects of drugs.

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by many authors such as Dews (1953), Saelens et al. (1968) Nakatsu and Owen, (1980). Recently developed devices allow to register not only general motor activity but also locomotion, rearing and the speed of locomotion (Barros et al. 1991; Ericson et al. 1991).

PROCEDURE

The rats are observed in a square open field arena (68 × 68 × 45 cm) equipped with 2 rows of 8 photocells, sensitive to infrared light, placed 40 and 125 mm above the floor, respectively. The photocells are spaced 90 mm apart and the last photocell in a row is spaced 25 mm from the wall. Measurements are made in the dark in a ventilated, sound-attenuating box. Interruptions of photocell beams can be collected by a micro-computer and the following variables can be evaluated:

- Motor activity: All interruptions of photo beams in the lower rows.
- Peripheral motor activity: Activation of photo beams in the lower rows, provided that the photo beams spaced 25 mm from the wall were also activated.
- Rearing: All interruption of the photo beams in the upper rows.
- Peripheral rearing: Interruption of photo beams in the upper rows, provided that the photo beams spaced 25 mm from the wall were also activated.
- Locomotion: Successive interruptions of photocells in the lower rows when the animal is moving in the same direction.
- Speed: The time between successive photo beam interruptions during locomotion collected in 0.1 s categories.

Adult male Sprague-Dawley rats with a weight between 280 and 320 g are used. Drugs are injected subcutaneously 10 to 40 min. before test. The rats are observed for 15 min whereby counts per min. are averaged for 3 min intervals.

EVALUATION

Dose-response curves can be obtained for sedative and stimulant drugs, whereby the various parameters show different results. The effects of various doses are compared statistically with the values of controls and among themselves.

CRITICAL ASSESSMENT OF THE METHOD

Measurement of several parameters in an open field device allows to differentiate between various types of sedative or stimulant drugs, but these differences can

E.1.2.3

Open Field Test

PURPOSE AND RATIONALE

Interruption of light beams as a measure of movements of rats or mice in a cage (“open field”) has been used

only be detected if dose-response curves are obtained for each parameter.

MODIFICATIONS OF THE METHOD

Besides interruption of light beams, devices based on capacitance systems such as Animex (Columbus Instruments, Ohio, USA) and Varimex have been developed and are widely used (Crunelli and Bernasconi 1979; Liu et al. 1985; Laviola G and Alleva E 1990; Honma et al. 1990; Magnus-Ellenbroek et al. 1993; Dauge et al. 1995; Petkov et al. 1995; Surmann et al. 1995; Gillies et al. 1996; Irifune et al. 1997; Ghelardini et al. 1998).

Nikodijevic et al. (1991) studied the behavioral effects of A₁- and A₂-selective adenosine agonists and antagonists in mice using a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) equipped with an IBM-compatible computer. The monitor included multiple activity monitor cages (40×40×30.5 cm), each of which was surrounded by horizontal and vertical sensors not detectable by the rodent.

Steiner et al. (1997) found that D₃ receptor deficient mice enter the center of an open field significantly more than their littermates suggesting an anxiolytic-like effect of the D₃ receptor mutation.

Vorhees et al. (1992) described a locomotor activity system for rodents. The system consists of a black, ventilated test chamber, internally lighted with a ceiling mounted video camera. The camera's image is transmitted to a contrast-sensitive tracker which maps the point of highest contrast and relays the digitalized coordinates to a PC. Dedicated software stores the information and simultaneously displays a map of the tracked subject.

Rex et al. (1996, 1998) described a modified open field test sensitive to anxiolytic drugs. Food-deprived rats were placed in one corner of the open field containing food in the center. The number of rats beginning to eat within the first 5 min was increased by known anxiolytic drugs.

Several authors used computerized systems, based on interruptions of infrared light beams, on magnetic field or on video-analysis such as the VideoMot2 system developed by TSE Systems, Bad Homburg, Germany, or systems available through Bilaney Consultants Ltd., St Julians, Sevenoaks, Kent, UK. (Sillaber et al. 1998; Wellmer et al. 2000; Kuzmin et al. 2003; Sienkiewicz-Jarosz et al. 2003; Strekalova et al. 2005).

Ströhle et al. (1997) mounted a video camera connected to a computer directly above six locomotor boxes (60×60 cm each). The distance traveled by the

rat in a 20-min interval was determined with a video digitizer (TSE Systems, Bad Homburg, Germany).

Spooren et al. (2000), Stobrawa et al. (2001), Heijtz et al. (2002, 2004), Abo-Salem et al. (2004), Bilkei-Gorzo et al. (2004) measured motor activity (locomotion and rearing) in several animals (rats or mice) simultaneously by means of a multi-box detection system (ActiMot; TSE Systems, Bad Homburg Germany). This system use individual photocell activity units (48 x 48 cm) connected to a control unit. Each unit consists of a base frame with two pairs of light-barrier strips (each with 16/32 pairs of infrared transmitter and receiver diodes set at a distance of 28/14 mm; XY-coordinate). An additional pair of light-barrier strips (Z-coordinate) is used to detect rearing activity. Data for the number and sequence of photocell interruptions were collected on a computer.

A novel method for counting spontaneous motor activity in rats was proposed by Masuo et al. (1997). In the "Supermex" system, a sensor detects the radiant body heat of an animal.

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E.1.2.4

Hole-Board Test

PURPOSE AND RATIONALE

The evaluation of certain components of behavior of mice such as curiosity or exploration has been attempted by Boissier et al. (1964) and Boissier and Simon (1964). They used an open field with holes on the bottom into which the animals could poke their noses. The “planche à trous” or “hole-board” test has become very well recognized and has been modified and automatized by many authors.

PROCEDURE

Mice of either sex (NMRI strain) with a weight between 18 and 22 g are used. The hole-board has a size of 40 × 40 cm. Sixteen holes with a diameter of 3 cm each are distributed evenly on the floor. The board is elevated so that the mouse poking its nose into the hole does not see the bottom. Nose-poking is thought to indicate curiosity and is measured by visual observation in the earliest description and counted by electronic devices in more recent modifications. Moreover, in the newer modifications motility is measured in addition by counting interruption of light beams. Usually, 6 animals are used for each dose and for controls. Thirty minutes after administration of the test compound the first animal is placed on the hole-board and tested for 5 min.

CALCULATION

The number of counts for nose-poking of treated animals is calculated as percentage of control animals.

CRITICAL ASSESSMENT OF THE METHOD

Poking the nose into a hole is a typical behavior of mice indicating a certain degree of curiosity. Evaluation of this component of behavior has been proven to be quite useful. Benzodiazepines tend to suppress nose-poking at relatively low doses.

MODIFICATIONS OF THE METHOD

A hole-poke measuring system is commercially available from TSE Systems, Bad Homburg, Germany.

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E.1.2.5

Combined Open Field Test

PURPOSE AND RATIONALE

The simultaneous determination of locomotion and curiosity by using a modification of the hole-board test and a photo-beam system has been proposed as a relatively simple test (Weischer et al. 1976). Several types of such equipment are commercially available.

PROCEDURE

Male mice (NMRI-strain) with an average weight of 30 g are used. Each animal is tested individually in an automated open-field box which consists of a black Plexiglas cage (35 × 35 × 20 cm) with a post (8 × 8 × 20 cm) in the center of the cage. Two evenly spaced photo cell beams perpendicular to the wall and 2 cm above the floor divide the box into 4 compartments. Every photo cell beam interruption is registered automatically as an activity count. Each wall of the cage contains 4 evenly spaced 2 cm diameter holes in a horizontal array 7 cm above the floor. A row of 4 photocell beams is mounted 1 cm outside of the holes and automatically records every exploratory nose-poke. Thirty min. after intraperitoneal

and 60 min. after oral administration of the test compound the animal is placed into the cage and the behavior recorded for a period of 5 min. Ten mice are used for each dose as well as for controls.

EVALUATION

Counts for motility (interruption of photo cell beams inside the cage) and for curiosity (interruption of photo cell beams outside the cage due to nose-poking) are recorded individually. The mean values of the treated groups are expressed as percentage of the control group. Using different doses, dose-response curves can be obtained.

CRITICAL ASSESSMENT OF THE METHOD

A dissociation between exploratory behavior and locomotion has been found with several drugs. Even well known stimulants can reduce exploratory behavior with a concomitant increase in locomotion. Depending on the dose, tranquilizers can reduce exploration without affecting locomotion. Due to the modifications of the equipment the results of different authors are often difficult to compare.

The limitations of photocell activity cages for assessing effects of drugs were discussed by Krsiak et al. (1970).

MODIFICATIONS OF THE TEST

Geyer (1982) described a similar device and reported different effects of amphetamine, caffeine, apomorphine and scopolamine. Adams and Geyer (1982) used this device to study the LSD-induced alterations of locomotor patterns and exploration in rats.

Geyer et al. (1986) described a behavioral monitor which was designed to assess the spatial and temporal sequences of locomotor movements in rats.

Other authors, e. g. Wolffgramm et al. (1988) used video recording of the movements of mice in the evening hours at low illumination and counted the locomotor activity as the number of field crossings and the exploratory activity as the number of rearings.

Ljungberg and Ungerstedt (1977) designed a test-box for the automatic recording of eight components of behavior in rats including compulsive gnawing induced by apomorphine.

Matsumoto et al. (1990) described a system to detect and analyze motor activity in mice consisting of a doughnut-shaped cage with 36 units of detectors radially arranged from the center of the cage. Each detector unit consisted of 4 pairs of photosensors (higher-, lower-, inner-, and outer-position sensors).

Schwarting et al. (1993) described a video image analyzing system for open-field behavior which measures turning behavior, thigmotactic scanning and locomotion in rats.

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E.1.2.6

EEG Analysis From Rat Brain by Telemetry

PURPOSE AND RATIONALE

Field potential analysis of freely moving rats by radio-electroencephalography from different brain areas has been developed as a sensitive method in pharmacology (Dimpfel et al. 1986, 1989, 1990, 1992; Kropf et al. 1991).

PROCEDURE

Male adult rats are implanted with four stainless steel electrodes, mounted on a base plate, into the frontal cortex, striatum, thalamus, and reticular formation. The plate carries a microplug for a four-channel radio-transmitter. During the experimental sessions, which

start 2 weeks after surgery, the transmitted field potentials are analyzed in real time using Fast Fourier Transformations. The resulting power density spectra are segmented into six frequency bands, each representing the integrated power over a certain frequency band.

EVALUATION

The data from three pre-drug periods of 15 min each are compared with those from continuously monitored 15-min postdrug periods.

MODIFICATIONS OF THE METHOD

De Simoni et al. (1990) developed a miniaturized optoelectronic system of telemetry for data obtained in freely moving animals by *in vivo* voltammetry, an electrochemical technique that uses carbon fiber microelectrodes to monitor monoamine metabolism and release continuously (Justice 1987).

Krügel et al. (2001) tested functional recovery after neuronal injury by P2 receptor blockade. The functional changes in the neuronal activity were recorded by telemetric EEG (TSE Systems, Bad Homburg, Germany).

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E.1.3

Tests for Muscle Coordination

E.1.3.1

Inclined Plane

PURPOSE AND RATIONALE

The method of Allmark and Bachinski (1949) using an inclined screen was originally developed for testing curare-like agents. Later on, it has been used by many authors (e.g. Randall et al. 1961) for testing compounds for muscle relaxant activity. The principle of an inclined plane has been used by Ther, Vogel and Werner (1959) for differentiating neuroleptics from other centrally active drugs. Rivlin and Tator (1977) also used an inclined plane to assess skeletal muscle relaxation.

PROCEDURE

The plane consists of two rectangular plywood boards connected at one end by a hinge. One board is the base, the other is the movable inclined plane. Two plywood side panels with degrees marked on their surface are fixed on the base. A rubber mat with ridges 0.2 cm in height is fixed to the inclined plane which is set at 65 degrees. Male mice (Charles River strain) with a body weight between 20 and 30 g are used. The test compound or the standard are administered to groups of 10 mice either i.p. or s.c. or orally. Thirty, 60 and 90 min thereafter, the mice are placed at the upper part of the inclined plane and are given 30 s to hang on or to fall off.

EVALUATION

The peak time is determined as the time at which a compound produces the maximum performance deficit. At this time interval, a range of doses is tested using 10 animals per group. ED_{50} values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method has been proven to be a simple assay for muscle relaxant activity. Although the muscle relaxant tests satisfy the criteria of sensitivity and relative potency compared with clinically effective doses, the effects of anxiolytics are not clearly differentiated from neuroleptics and even from neurotoxic compounds.

MODIFICATIONS OF THE METHOD

Instead of an inclined wooden board, an inclined screen has been used by Randall et al. (1961) and Simiand et al. (1989).

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E.1.3.2**Chimney Test****PURPOSE AND RATIONALE**

The “test de la cheminée” has been introduced by Boissier et al. (1960) as a simple test for tranquilizing and muscle relaxant activity.

PROCEDURE

Male mice (CD1, Charles River) weighing between 16 and 22 g are used in groups of 10 animals per dose. Pyrex-glass cylinders 30 cm long are required. The internal diameter varies with the animal's weight: for mice weighing 16 to 18 g, the diameter is 22 mm, for mice weighing 18 to 20 g, 25 mm; for mice weighing 20 to 22 g, 28 mm. Each tube has a mark 20 cm from its base. Initially, the tube is held in a horizontal position. At the end of the tube, near the mark, a mouse is introduced with the head forward. When the mouse reaches the other end of the tube, toward which it is pushed if necessary with a rod, the tube is moved to a vertical position. Immediately, the mouse tries to climb backwards and performs coordinated movements similar to an alpinist to pass a chimney in the mountains. This gave the name for the test. The time required by the mouse to climb backwards out at the top of the cylinder is noted.

EVALUATION

The ED_{50} (with 95% confidence limits), the dose for which 50% of the animals fail to climb backwards out of the tube within 30 s, is calculated by log-probit analysis.

CRITICAL ASSESSMENT OF THE METHOD

The chimney test can be used as an additional test with other tests determining muscle relaxant activity.

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E.1.3.3**Grip Strength****PURPOSE AND RATIONALE**

The test is being used to assess muscular strength or neuromuscular function in rodents which can be influenced not only by sedative drugs and muscle relaxant compounds but also by toxic agents.

PROCEDURE

The method was described as ‘test de l'agrippement’ by Boissier and Simon (1960). Male or female mice with an average weight of 22 g are used. In a preliminary experiment the animals are tested for their normal reactivity. The animals are exposed to a horizontal thin threat or metallic wire suspended about 30 cm into the air, which they immediately grasp with the forepaws. The mouse is released to hang on with its forelimbs. Normal animals are able to catch the threat with the hind limbs and to climb up within 5 s. Only animals who fulfill this criterion are included into the experiment. Ten mice are used in the control group and in the experimental groups. After oral or subcutaneous administration the animals are tested every 15 min. Animals which are not able to touch the threat with the hind limbs within 5 s or fall off from the threat are considered to be impaired. The test is continued for 2 h. The animals are observed for their behavior in the cages. Only if their behavior and their motility in the cage seem to be normal the disturbance of the grasping reflex can be considered as caused by central relaxation.

EVALUATION

The percentage of animals losing the catching reflex is calculated. By use of different doses, ED_{50} -values are calculated. Likewise, time-response curves can be established.

CRITICAL ASSESSMENT OF THE METHOD

Only simultaneous observation of the animals under normal conditions gives the possibility to distinguish between central relaxation and toxic effects on neuromuscular function.

MODIFICATIONS OF THE METHOD

Kondziella (1964) described a method for the measurement of muscular relaxation in mice based on the capacity of hanging from a horizontal griddle.

Meyer et al. (1979) described a technique to measure the fore- and hind limb grip strength of rats and mice. The apparatus consists of an adjustable trough and a push-pull strain gauge with a triangular brass ring which is grasped by the animal with its forelimbs. The animal is pulled on the tail until the grip is broken. The animal continues to be pulled along the trough until the hind limbs grasp a T-shaped bar being also attached to push-pull strain gauge. The trial is completed when the grip of the hind limbs is also broken. Fore- and hind limb strength are measured. Dose-response curves could be established with various doses of chlordiazepoxide and phenobarbital.

Barclay et al. (1981) described the tight rope test for testing performance in mice.

Simiand et al. (1989) used a test originally described by Fleury (1957). A mouse held by the tail is placed on a small metallic grid which the animal gripped with its forepaws. The grid is then loaded with weights until the mouse could no longer support the weight of the grid. The endpoint is the maximal weight supported by the animal at least for 2 s. ED_{50} -values can be calculated for various centrally active skeletal muscle relaxants such as the benzodiazepines.

Deacon and Gardner (1984) described the pull-up test in rats. A rat is held by its hind legs in an inverted position. The time taken by the rat to pull itself up and grasp the hand of the experimenter is used as the test parameter.

Novack and Zwolshen (1983) tested muscle relaxants in various models, such as morphine-induced rigidity in rats, decerebrate rigidity in cats and the polysynaptic linguomandibular reflex in cats.

Montag-Sallaz and Montag (2003), Anderson et al. (2004), and Baier et al. (2004) used a Grip Strength Meter apparatus, designed by TSE Systems, Bad Homburg, Germany. If the animal releases the grip then the maximum force is shown on a digital display of a connected control unit.

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E.1.3.4**Rotarod Method****PURPOSE AND RATIONALE**

The test is used to evaluate the activity of drugs interfering with motor coordination. In 1956, Dunham and Miya suggested that the skeletal muscle relaxation induced by a test compound could be evaluated by testing the ability of mice or rats to remain on a revolving rod. This forced motor activity has subsequently been used by many investigators. The dose which impairs the ability of 50% of the mice to remain on the revolving rod is considered the endpoint.

PROCEDURE

The apparatus consists of a horizontal wooden rod or metal rod coated with rubber with 3 cm diameter attached to a motor with the speed adjusted to 2 rotations per minute. The rod is 75 cm in length and is divided into 6 sections by plastic discs, thereby allowing the simultaneous testing of 6 mice. The rod is in a height

of about 50 cm above the table top in order to discourage the animals from jumping off the roller. Cages below the sections serve to restrict the movements of the animals when they fall from the roller. Male mice (CD-1 Charles River strain) with an weight between 20 and 30 g undergo a pretest on the apparatus. Only those animals which have demonstrated their ability to remain on the revolving rod for at least 1 minute are used for the test. The test compounds are administered intraperitoneally or orally. Thirty minutes after intraperitoneal or 60 min after oral administration the mice are placed for 1 min on the rotating rod. The number of animals falling from the roller during this time is counted.

Using different doses, ED_{50} values can be calculated. Moreover, testing at various time intervals, time-response curves can be obtained.

CALCULATION

Percent animals falling from the rotarod within the test period is calculated for every drug concentration tested. ED_{50} is defined as the dose of drug at which 50% of the test animals fall from the rotarod.

CRITICAL ASSESSMENT OF THE TEST

Many central depressive drugs are active in this test. Benzodiazepines, such as diazepam and flurazepam, have ED_{50} values below 1 mg/kg i.p. The activity of neuroleptics, such as chlorpromazine or haloperidol, is in the same range. In this way, the test does not really differentiate between anxiolytics and neuroleptics but can evaluate the muscle relaxant potency in a series of compounds such as the benzodiazepines. Moreover, the test has been used in toxicology for testing neurotoxicity.

MODIFICATIONS OF THE METHOD

A comparison of the rotarod method in rats with other tests, such as blockade of morphine-induced rigidity in rats, decerebrate rigidity in cats, and polysynaptic-monosynaptic reflex preparations in cats was published by Novack and Zwolshen (1983).

Capacio et al. (1992) used the accelerating rotarod to assess motor performance decrement in rats after administration of candidate anticonvulsant compounds in nerve agent poisoning.

The accelerating rotarod system (TSE Systems, Bad Homburg, Germany) was also used by Augustin et al. (2001), Hosseinzadeh and Asl (2003), Dere et al. (2003), and Vitali and Clarke (2004).

Rozas et al. (1997) described a drug-free rotarod test that was used to evaluate the effects of unilateral

6-hydroxydopamine lesions, nigral grafts, and subrotational doses of apomorphine. The rotarod unit was automated and interfaced with a personal computer allowing automatic recording of the time that each rat was able to stay on the rod at different rotational speeds.

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E.1.3.5

Treadmill Performance

PURPOSE AND RATIONALE

Treadmill experiments have been used in pharmacology and physiology for various purposes:

1. To evaluate the influence of drugs on endurance (Snider et al. 1983; Matthew et al. 1988; Ahlenius et al. 1977a, 1997b; Soares et al. 2003; Minami et al. 2004).
2. To study muscle metabolism (Balon and Nadler 1997; Zhou and Dohm 1997; Juel 1998; Foianini et al. 2000; Gosmanov et al. 2002; Saengsirisuwan et al. 2002; Steinberg et al. 2004).
3. To study influence on the cardiovascular system (Koller et al. 1995; Roth et al. 1998; Spier et al. 1999; Sun et al. 1994, 2002; Villanueva et al. 2003).

4. To study the effect of diet (Oudot et al. 1996) and of circadian rhythm (Marchant and Mistlberger 1996).

PROCEDURE

Minami et al. (2004) studied the effect of a high-salt diet or chronic captopril treatment on exercise capacity in normotensive rats. Male Sprague Dawley rats were submitted to stepwise increasing exercise on a motor treadmill at a speed of 10, 20 and 30 m/min, 0-grade incline, for 4 min with 2-min rest intervals. Exercise was stopped when rats were unable to continue running with exhaustion despite contact with a shock at the rear of the treadmill belt. Mean arterial pressure (MAP) and heart rate (HR) were recorded continuously at rest and during the three different intensities of exercise.

EVALUATION

Values obtained for MAP and HR during the 10 s before the beginning of exercise, when all values reached a stable state, and during the last 10 s at each exercise level were used for comparison. All results are expressed as mean \pm SEM. One-way ANOVA was used to assess significant differences in means across groups at rest and at each exercise level.

TREADMILL SYSTEMS

A fully computerized electronically controlled treadmill system for small laboratory animals is provided by TSE Systems, Bad Homburg, Germany: The apparatus basically consists of a rotating running belt driven by a servo-controlled motor, which provides precise operator-defined tread speed. Separating panels divide the running surface into separate exercise lanes each suited for an individual animal. The floor grids can be used to apply an electric stimulus of variable length and intensity. The incline of the running surface can be steplessly adjusted to control the labor of the animal.

Columbus Instruments, Columbus, OH, offers the animal treadmill Exer 3/6, which is a general purpose treadmill for three rats or 6 mice utilizing a single belt construction with dividing walls suspended over the tread surface supplied with or without a stimulus assembly. Speed of the belt can be adjusted in the range of 6–100 m/m.

MODIFICATIONS OF THE METHOD

Treadmill experiments in **cats** were performed by Douglas et al. (1993).

Nakai et al. (2003) and Liu et al. (2004) used treadmill experiments to study walking dysfunction in the **rat neuropathic intermittent claudication model**.

In this model, male Sprague Dawley rats were anesthetized with pentobarbital. L4–L6 vertebrae were surgically exposed and two small holes were drilled, one between L4–L5 and the second between L6–L6 intervertebral spaces. Two rectangular silicone rubber strips (1.25 mm \times 4 mm, thickness: 1 mm) were then placed in the L4–L6 epidural spaces. The incision was closed in two layers and penicillin was administered systemically.

Antri et al. (2005) used treadmill experiments to study long-lasting recovery of locomotor function in chronic spinal rats following combined pharmacological stimulation of serotonergic receptors with 8-OHDPAT and quipazine.

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- kg i.p.). For the flexor reflex a hindpaw is stimulated with a pair of fine subcutaneous needle electrodes (5 square-wave shocks at 500 Hz, 0.2 ms duration and 3.0 reflex threshold). Electromyographic recordings are made with a pair of fine needle electrodes inserted into the ipsilateral tibialis muscle. Seven consecutive electromyographic recordings are amplified, and band-pass filtered (8–10 Hz), collected at a sample rate of 10 Hz, averaged and evaluated using a Signal Averager (CED, Cambridge, UK) on an IBM-compatible personal computer. Ten consecutive responses are averaged before and 20 min after i.p. application of drug.
- For stimulation of the Hoffmann reflex a pair of needle electrodes is transcutaneously inserted into the surrounding of the tibial nerve (single square-wave shocks, 0.2 ms duration at 2.0 threshold). Electromyographic recordings are made with a pair of skin clip surface electrodes from the plantar foot muscle. Low intensity electrical stimulation of the tibial nerve elicits a reflex response similar to the human Hoffmann (H) reflex, which has been attributed to monosynaptic excitation of spinal α -motoneurons predominantly by primary muscle spindle afferent fibers. With increasing stimulus strength, the H-wave is preceded by an electromyographic wave, the M wave, which is due to a direct excitation of axons of α -motoneurons.
- In all reflex experiments, values measured after solvent or drug application are expressed as a percentage of the corresponding pre-injection value.

EVALUATION

Statistical evaluation of group differences is performed using the Mann-Whitney U-test. Statistical analysis for dose dependency of drug effects is carried out by the Kruskal-Wallis test.

MODIFICATIONS OF THE METHOD

Ono et al. (1990), Farkas and Ono (1995), Hasegawa and Ono (1996), Otsu et al. (1998) recorded spinal reflexes from spinalized and non-spinalized rats anesthetized with α -chloralose and urethane. Laminectomy was performed in the lumbo-sacral region. Ventral and dorsal roots of the segments L4 and L5 were isolated. A skin pouch was formed at the site of the dissection to cover the exposed tissues with liquid paraffin kept at 36°C. The dorsal root of L5 was placed on bipolar silver wire electrodes for stimulation (0.2 Hz, 0.05 ms, supramaximal). The ipsilateral ventral root of L5 and the dorsal root of L4 were placed on bipolar wire electrodes for recording. Monosynaptic and polysynaptic reflexes and dorsal root-dorsal root reflexes were evoked in the L5 ventral root and in the L4 dorsal

E.1.3.6

Influence on Polysynaptic Reflexes

PURPOSE AND RATIONALE

Inhibition of polysynaptic reflexes is considered to be the major mode of action of muscle relaxants. Polysynaptic transmission can be measured by the flexor reflex of the hindpaw in anesthetized rats, whereas the monosynaptic Hoffmann reflex is measured by electromyographic recordings from the plantar foot muscle (Block and Schwarz 1994; Schwarz et al. 1994, 1996).

PROCEDURE

Male Wistar rats (250–280 g) are anesthetized with urethane (400 mg/kg i.p.) and α -chloralose (80 mg/

root, respectively. These reflex potentials were amplified, displayed on an oscilloscope and averaged 8 times by an averaging computer.

Turski and Stephens (1993) recorded monosynaptic Hoffman reflexes in NMRI mice anesthetized with 80 mg/kg α -chloralose i.p. +400 mg/kg urethane i.p. The tibial nerve was stimulated with single square-wave pulses, 0.2 ms duration at 1.2–1.6 times the nerve threshold. Electromyogram recordings were made with a pair of skin clip surface electrodes from the plantar foot muscle. For recording polysynaptic flexor reflexes, the tibial nerve was stimulated with five square-wave pulses at 500 Hz, 0.2 ms duration at 3.0 times the nerve threshold. Electromyogram recordings were made with a pair of wire electrodes inserted percutaneously into the ipsilateral tibial muscle.

Furthermore, these authors used **genetically spastic rats**. A mutant strain of Wistar rats, which carries an autosomal recessive gene defect, is characterized by a progressive paresis of the hindlimbs with increased tone on the extensor muscles (Pittermann et al. 1976). This genetically determined syndrome of spasticity in the rat permits quantitative evaluation of the effect of drugs on muscle tone by recording activity in the electromyogram from a hindlimb extensor muscle (Klockgether et al. 1985; Turski et al. (1990).

Farkas et al. (1989), Tarnava et al. (1989) studied the effects of drugs on the reflex potentials evoked by afferent nerve stimulation and recorded from the spinal roots in unanesthetized spinal cats. An analog integrating method was used for quantitative evaluation of the reflex potentials. The amplified and band-pass filtered signals from the ventral root (monosynaptic reflex and polysynaptic reflex) and from the dorsal root (dorsal root reflex and dorsal root potential) were fed into signal-selectors, which transmitted the input signals only within the chosen post-stimulus intervals. Thus, the various components of the reflex potentials were separated according to their latencies.

Shakitama et al. (1997) recorded ventral root reflex potential in anesthetized rats and ventral and dorsal root potentials in anesthetized intact and spinalized cats.

Suzuki et al. (1995) studied the recovery of reflex potentials after spinal cord ischemia produced by occlusion of the thoracic aorta and the bilateral internal mammary arteries for 10 min in cats.

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E.1.3.7

Masticatory Muscle Reflexes

PURPOSE AND RATIONALE

The temporomandibular joint dysfunction syndrome, characterized by pain and clicking in the temporomandibular joint and limitation of function, involves a condition caused by hypertonia combined with para-

functional habits, such as clenching or grinding of teeth and hyperreflexia (Laskin and Block 1986) which may be treated by muscle relaxants. Ozawa et al. (1996) studied the effects of a centrally acting muscle relaxant on *masticatory* muscle reflexes in rats. Both monosynaptic and polysynaptic reflexes can be studied using this model.

PROCEDURE

For recording the **monosynaptic tonic vibration reflex** of the masseter muscle, Wistar rats are anesthetized with ether, intubated with a tracheal cannula and fixed into a stereotactic apparatus. Decerebration is performed by radiofrequency lesion of the midbrain using a Lesion Generator (Radionics, RFG-4, USA) and a lesioning electrode inserted into the midbrain. After lesioning, ether anesthesia is discontinued. The tonic vibration reflex of the masseter muscle, recorded as electromyogram, is induced every 10–12 s by a sinusoidal vibration (100–500 Hz, 2 s) which is applied to the mandibula, and is delivered by a vibration generator driven by a low frequency oscillator and an amplifier. The evoked electromyogram is amplified by a biophysical amplifier and recorded on a thermal array recorder. The root mean square of the electromyogram is also recorded through an integrator.

For recording the **polysynaptic jaw opening reflex**, the animals are anesthetized by intraperitoneal pentobarbital-Na (50 mg/kg), fixed into position on their back and intubated with a tracheal cannula. Intrapulpal stimulation (0.5 Hz, 0.2 s in pulse duration, supramaximal intensity) delivered by an electrical stimulator is performed via a dental reamer inserted into the dental pulp of the mandibula. The jaw opening reflex recorded as phasic component of the electromyogram evoked in the ipsilateral digastric muscle is amplified by a biophysical amplifier and recorded on a thermal array recorder.

For recording of the **polysynaptic tonic periodontal masseteric reflex** (Funakoshi and Amano 1974) the animals are anesthetized by intraperitoneal injection of pentobarbital-Na (35 mg/kg), which is supplemented as required, intubated with a tracheal cannula and fixed onto a stereotaxic apparatus. The maxillary incisor is stimulated by pressing for 5 s every 5 min using a vibration generator driven by a trapezoid generator. The electromyogram responses to this stimulation are amplified by a biophysical amplifier and recorded on a thermal array recorder. The evoked electromyogram is transformed into square-wave pulses, fed into a staircase generator and recorded on a thermal array recorder.

EVALUATION

The significance of differences between the control and the drug-treated groups is evaluated with Dunnett's test.

MODIFICATIONS OF THE METHOD

Boucher et al. (1993) performed microinfusions of excitatory amino acid antagonists into the trigeminal sensory complex of freely moving rats while recording the long latency jaw opening reflex elicited by electrical stimulation of the dental pulp.

Bakke et al. (1998) studied in anesthetized rats neurokinin receptor mechanisms in the increased jaw muscle activity which can be evoked by injection of the small fiber excitant and inflammatory irritant mustard oil into the temporomandibular joint region.

Alia et al. (1998) performed intra-oral administration of a NK₁ antagonist in freely moving guinea pigs during recording the short- (6–10 ms) and long-latency (18–26 ms) jaw-opening reflex elicited by electrical stimulation of the lower incisor tooth pulp.

Huopaniemi et al. (1988) determined the threshold of the tooth-pulp evoked jaw-opening reflex after naloxone in barbiturate-anesthetized cats.

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E.2

Tests for Anxiolytic Activity

E.2.0.1

General Considerations

Definitions in psychopharmacology have been coined by the activity of special compounds or chemical

classes found in patients. This is not only true for the term “neuroleptic” but also for the term “anxiolytic”. Other terms have been “ataractic” or “psycholeptic”. Anxiolytics are derived from “tranquilizers”, such as meprobamate which was used widely until the advent of benzodiazepines. The property which these drugs have in common is the alleviation of anxiety, thus explaining the term “anxiolytic”. These agents are used for the relatively minor disorders of the nonpsychotic or neurotic type, whereas the antipsychotic agents (phenothiazines, butyrophenones) are given mainly to combat the more severe psychotic or schizophrenic reactions. Thus, the terms anti-anxiety and antipsychotic indicate a qualitative distinction in the clinical use and mode of action of the drug. Pathological anxiety in man has been defined by its interference with normal functions, by manifestations of somatic disorders, emotional discomfort, interference with productivity at work, etc. This complex characterization of anxiety in man already indicates the difficulties to find appropriate pharmacological models. Therefore, several tests have to be performed to find a spectrum of activities which can be considered to be predictive for therapeutic efficacy in patients. For *in vivo* studies, most investigators use a battery of anticonvulsive tests, anti-aggressive tests and evaluation of conditioned behavior.

Most of the actions of benzodiazepines are thought to be mediated by potentiation of γ -amino-butyric acid (GABA). Two subtypes of GABA receptors (GABA_A and GABA_B) have been described. Moreover, specific binding sites for benzodiazepines have been discovered near these GABA receptors in various areas of the brain. These sites occur in a macromolecular complex that includes GABA-receptors, benzodiazepine receptors and receptors for other drugs, and a chloride channel. The benzodiazepines potentiate the neurophysiological actions of GABA at the chloride ion channel by increasing the binding of GABA to GABA_A receptors. This implies that the GABA_A receptor is involved in anxiety and that its direct activation would have an anxiolytic effect. Based in these findings various *in vitro* tests have been developed.

More recently, research has focused on the therapeutic potential of blocking excitatory amino acids – in particular glutamate. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). Some methods are described in Sect. E.3 Anti-epileptic activity.

Serotonin may play a role in anxiety, since treatment with drugs that reduce serotonergic function,

including benzodiazepines, have anxiolytic effects in animal models (Dourish et al. 1986). Several subtypes of serotonin receptors have been elucidated, e. g. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, 5-HT₂, 5-HT₃. Further differentiation is underway. 5-HT_{1A}, 5-HT_{1B}, and 5-HT₃ receptors are considered to be involved in the effect of anti-anxiety and novel antipsychotic drugs (Peroutka 1988; Costall et al. 1988). Some *in vitro* methods are described in Sect. E.6 Antidepressant activity and Sect. E.5 Neuroleptic activity.

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E.2.1

In Vitro Methods

E.2.1.1

GABA Receptor Binding

E.2.1.1.1

General Considerations

γ -Aminobutyric acid (GABA) is known to be an important inhibitory neurotransmitter in the brain. Abnormalities in the GABA system have been found in neurological and psychiatric diseases such as Hunt-

ingdon's chorea, anxiety, panic attacks, schizophrenia, and epilepsy. GABA is also implicated in the mechanism of benzodiazepines and related central nervous system (CNS) drugs.

GABA receptors have been divided into GABA_A, GABA_B and GABA_C receptor subtypes which by themselves form receptor families (Enna and Möller 1987; Matsumoto 1989; Möhler 1992).

Without doubt, GABA is the most important inhibitory transmitter in the CNS. One may expect important new developments from attempts to influence the GABAergic system specifically by synthetic compounds. In general, GABA_A receptor agonists are CNS depressants, muscle relaxants, and possess some noiceptive properties, whereas the receptor antagonists are convulsants. Most benzodiazepine-binding sites seem to be associated with GABA_A receptors combined with a chloride channel. Among GABA_B receptor agonists, baclofen has achieved success in clinical use. Results of ongoing research with receptor subtypes will result in the development of new therapeutic agents.

Studies by Urwyler et al. (2001) demonstrated that the GABA_B receptor can be modulated in an allosteric manner. These compounds are not receptor agonists but they appear to act on the heptahelical region of GABA_{B2} to enhance the action of the receptor agonists GABA and baclofen.

GABA_C receptors are ligand-gated chloride channels that are present in many parts of the brain including the superior colliculus, cerebellum, hippocampus, and, most prominently, the retina (Ragozzino et al. 1996; Bormann 2000; Qian and Ripps 2001; Chehib 2004; Schlicker et al. 2004; Yang 2004; Pan et al. 2005; Wang and Slaughter 2005).

The formation and plasticity of GABAergic synapses, their physiological mechanisms and pathophysiological implications are discussed by Fritschy and Brünig (2003).

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E.2.1.1.2

In Vitro Assay for GABAergic Compounds: [³H]-GABA Receptor Binding

PURPOSE AND RATIONALE

Radiolabeled GABA is bound to synaptic membrane preparations of mammalian brain. The labeling of the synaptic receptor with ³H-GABA requires careful attention to possible interference from nonsynaptic binding since ³H-GABA can also bind nonspecifically to plasma membranes. The most prominent of which is the sodium dependent binding of GABA to brain membranes, a process which appears to be associated with the transport (uptake) sites of GABA. Sodium-independent binding of ³H-GABA has characteristics consistent with the labeling of GABA receptors. In addition, the relative potencies of several amino acids in competing for these binding sites parallel their abilities to mimic GABA neurophysiologically. Therefore, the sodium-independent binding of ³H-GABA provides a simple and sensitive method to evaluate compounds for GABA-mimetic properties.

PROCEDURE

Reagents

- 0.05 M Tris-maleate buffer (pH 7.1)
- 6.05 g of Tris-base are dissolved in distilled water and made up to 1000 ml. 5.93 g of Tris-maleate are dissolved in 500 ml of water. The 0.05 M Tris

maleate, pH 7.1 buffer is prepared by slowly adding Tris maleate to the Tris-base solution until the pH reaches 7.1.

- 0.32 M Sucrose: 109.5 g of sucrose are dissolved in distilled water and filled up to 1000 ml. The solution is stored at 4°C.
- ³H-GABA (specific activity approximately 40 Ci/mmol) is made up to a concentration of 780 nmol in distilled water and 20 µl is added to each test tube (yielding a final concentration of 15 nmol in the assay). Isoguvacine or muscimol is prepared by dissolving 8.35 mg of isoguvacine or 6.40 mg of muscimol in 10 ml water. Twenty µl of these solutions when added to 1 ml of incubation medium give a final concentration of 0.1 mM isoguvacine or muscimol.
- Test drugs: 1 mM stock solutions are initially prepared. These are serially diluted to the required concentrations prior to the addition to the incubation mixture. Final concentrations are usually from 2×10^{-8} to 1×10^{-5} M.

Tissue Preparation

Male Charles-River rats (100–150 g) are decapitated and their whole brains rapidly removed and homogenized in 15 vol of ice-cold 0.32 M sucrose. The homogenate is centrifuged at 1000 g for 10 min. The pellet (nuclear fraction) is discarded and the supernatant fluid is recentrifuged at 20,000 g for 20 min. The supernatant is discarded and the crude mitochondrial pellet is resuspended in 15 vol distilled water using a Tekmar homogenizer. The suspension is centrifuged at 8000 g for 20 min. The supernatant is collected and used to carefully resuspend, using a gentle squirling motion, the pellet's soft, upper, buffy layer. This suspension is then centrifuged at 48,000 g for 20 min. The final crude synaptic membrane pellets are resuspended (without homogenization) in 15 volumes of distilled water and centrifuged at 48,000 g for 20 min. The supernatant is discarded, and the centrifuge tubes containing the pelleted membranes are capped with parafilm and stored frozen at -70°C.

Assay Procedure

A frozen membrane pellet from one whole rat brain is resuspended in 15 volumes of 0.05 M Tris-maleate buffer (pH 7.1) by homogenization at 4°C. Triton X-100 is added to a final concentration of 0.05%. This suspension is then incubated at 37°C for 30 min followed by centrifugation at 48,000 g for 10 min. The supernatant is discarded and the pellet resuspended by homogenization in the same volume of 0.05 M Tris-

maleate buffer (pH 7.1) at 4°C. The preincubation with Triton enhances specific GABA receptor binding while lowering non-specific binding.

For the standard Na-independent ³H-GABA binding assay procedure, aliquots of the previously frozen, Triton treated crude synaptic membranes are incubated in triplicate at 4°C for 5 min in 0.05 M Tris-maleate buffer (pH 7.1) containing 15 nM ³H-GABA alone or in the presence of 0.1 mM isoguvacine or muscimol, or the test drug.

The procedure is as follows:

- 1 ml of the 0.05 M Tris-maleate homogenate
- 20 µl of ³H-GABA
- 20 µl of test drug or 20 ml of 0.1 mM isoguvacine or muscimol.

After incubation at 4°C for 5 min, the reaction is terminated by centrifugation for 15 min at 5000 rpm. The supernatant fluid is aspirated and the pellet washed twice with 1 ml of the Tris-maleate buffer. Two ml of liquiscint are added to each tube which is then vigorously vortexed. The contents of the tubes are transferred to scintillation vials, and the tubes rinsed with an additional 2 ml of cocktail. An additional 6 ml of liquiscint are added to each scintillation vial. The radioactivity is measured by liquid scintillation photometry.

EVALUATION

Specific ³H-GABA binding is defined as the radioactivity which can be displaced by a high concentration of unlabeled GABA and is obtained by subtracting from the total bound radioactivity the amount of radioactivity bound in the presence of 0.1 mM isoguvacine. Results are converted to percent of specifically bound ³H-GABA displaced by a given concentration of test drug. *IC*₅₀ values with 95% confidence limits are then obtained by computer derived linear regression analysis.

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E.2.1.1.3

GABA_A Receptor Binding

PURPOSE AND RATIONALE

Two subtypes of GABA receptors have been identified:

1. GABA_A receptor for which muscimol is the typical agonist, whereas bicuculline, picrotoxin, and SR 95531 are antagonists, and
2. GABA_B receptor, for which baclofen is the typical agonist.

The GABA_A receptor directly gates a Cl⁻ ionophore and has modulatory binding sites for benzodiazepines, barbiturates, neurosteroids and ethanol. By contrast, GABA_B receptors couple to Ca²⁺ and K⁺ channels via G proteins and second messenger systems; they are activated by baclofen and are resistant to drugs that modulate GABA_A receptors.

Several subtypes of GABA_A receptors have been identified by ligand binding studies (Kleingoor et al. 1991; Turner et al. 1992; Gusti et al. 1993). Molecular biology techniques revealed the GABA_A receptor to be assembled as a pentameric structure from different subunit (α , β , γ and δ subunit) families making it possible that a very large number of such heteromeric GABA_A receptors exist in the mammalian central and peripheral nervous system (Krogsgaard-Larsen et al. 1994; Lambert et al. 1995; Smith and Olsen 1995; Costa 1998). A total of 19 genes encoding GABA_A receptor subunits are known, while several additional isoforms can occur as splicing variants of some of these (Barnard 1998). Probably more than 500 distinct GABA_A receptor subtypes exist in the brain (Sieghart 2000). Sequences of six α , three β , three γ , one δ , three ρ , one ϵ , one π and one θ GABA_A receptor subunits have been reported in mammals (Barnard 2000; Alexander et al. 2001). More insight into the pharmacological functions of GABA_A receptor subtypes is expected from studies in gene-knockout mice and by knock-in point mutations (Rudolph et al. 2001).

GABA_C receptors were described as a pharmacologically distinct group by Bormann and Feigenspan (1995), Johnston (1996), Bormann (2000), Zhang et al. (2001). These receptors are Cl⁻ pores that are insensitive to both bicuculline and baclofen. An IUPHAR

Committee has recommended in 1998, that the term GABA_C should be avoided and classified bicuculline- and baclofen-insensitive GABA receptors as a minor subspecies of GABA_A receptors of the 'AO' type.

Subtypes of GABA_A receptors were classified on the basis of subunit structure and receptor function by Barnard et al. (1998).

In rat brain membranes, only the GABA_A receptor is labeled with the GABA_A selective radioligand ³H-SR 95531 in the given concentration range. The assay allows specifically the estimation of the test drug's binding characteristics to the GABA_A receptor subpopulation.

PROCEDURE

Materials

Radioligand: ³H-SR 95531 (³H-2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide (New England Nuclear, Boston)

Membrane Preparation

Rats are killed by decapitation, the brains are quickly dissected and after separation from the cerebellum placed into ice-cold sucrose solution. The brains (approximately 20 g wet weight) are then homogenized in a glass Teflon potter (1 g brain weight/15 ml 320 mM D(+)-sucrose solution), and centrifuged at 1000 g at 4°C for 10 min. The pellets are discarded and the supernatants centrifuged at 20,000 g for 20 min. The resulting supernatants are discarded and the pellets are lysed by hypoosmotic shock (addition of 20 volumes of ice-cold bidistilled water). After homogenization in a glass Teflon homogenizer, the suspension is stirred under cooling for 20 min, and centrifuged at 48,000 g for 20 min. The resulting pellets are resuspended in ice-cold bidistilled water, the suspension is stirred and recentrifuged as before. The final pellets are resuspended in the incubation buffer (50 mM Tris-HCl and 100 mM MgCl₂ × 6 H₂O, pH 7.4) corresponding to 1 g brain wet weight/1 ml buffer. The membrane suspension is immediately stored in aliquots of 1 ml at -20°C. Protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and diluted 1:20 with bidistilled water. After stirring for 10 min, the membrane suspension is centrifuged at 50,000 g for 10 min. The resulting pellets are resuspended in ice-cold incubation buffer, yielding a membrane suspension with a protein content of 1 mg/ml.

Assay

For each concentration, assays are performed in triplicate. The total volume of each incubation sample is 200 μ l (microtiter plates).

Saturation Experiments

Total binding:

- 50 ml ^3H -SR 95531 (12 concentrations, 2×10^{-9} to 1×10^{-7} M)
- 50 ml incubation buffer

Non-specific binding:

- 50 ml ^3H -SR 95531 (4 concentrations, 2×10^{-9} to 1×10^{-7} M)
- 50 ml (+) bicuculline (10^{-4} M)

Competition Experiments

- 50 ml ^3H -SR 95531 (1 constant concentration, $8\text{--}10 \times 10^{-9}$ M)
- 50 ml incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10} to 10^{-3} M)

The binding reaction is started by adding 100 μ l membrane suspension per incubation sample. The samples are incubated for 30 min at 4°C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane-bound radioactivity is separated from the free radioactivity. Filters are washed immediately with approximately 20 ml ice-cold rinse buffer (50 mM Tris HCl, pH 7.4) per sample. The membrane-bound radioactivity is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^3H -SR 95531 versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D^3\text{H} \times \text{IC}_{50}}{K_D^3\text{H} + [^3\text{H}]}$$

IC_{50} = concentration of the test drug, which inhibits 50% of specifically bound ^3H -SR 95531 in the competition experiment.

$[^3\text{H}]$ = concentration of ^3H -SR 95531 in the competition experiment.

$K_D^3\text{H}$ = dissociation constant of ^3H -SR 95531, determined from the saturation experiment.

The K_i -value of the test drug is that concentration, at which 50% of the receptors are occupied by the test drug.

MODIFICATIONS OF THE METHOD

Binding to the agonist site of the GABA_A receptor can be measured with [^3H]muscimol (Snodgrass 1978; Williams and Risley 1979; Martini et al. 1983).

A membrane fraction of whole brains (except cerebellum) from male Wistar rats is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1 nM [^3H]muscimol for 10 min at 0°C. Non-specific binding is estimated in the presence of 100 nM muscimol. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [^3H]muscimol.

The GABA_A receptor chloride channel can be studied by binding with [^3H]t-butylbicycloorthobenzoate ([^3H]TBOB) (Schwartz and Mindlin 1988; Lewin et al. 1989).

A membrane fraction of whole brains (except cerebellum) from male Wistar rats is prepared by standard techniques. 0.4 mg of membrane preparation is incubated with 3 nM [^3H]TBOB for 15 min at 15°C. Non-specific binding is estimated in the presence of 200 μ M picrotoxin. Membranes are filtered and washed 3 times and the filters are counted to determine [^3H]TBOB specifically bound.

Cromer et al. (2002) presented a model for the extracellular, ligand-binding domain of the GABA_A receptor that is based on the structure of a soluble acetylcholine-binding protein.

Kittler and Moss (2003) discussed modulation of GABA_A receptor activity by phosphorylation and receptor trafficking and the implications for the efficacy of synaptic inhibition.

Lambert et al. (2003) reviewed neurosteroid modulation of GABA_A receptors with special emphasis on pregnane steroids.

Lüscher and Keller (2004) described regulation of GABA_A receptor trafficking, channel activity, and functional plasticity of inhibitory synapses.

Macdonald et al. (2004) reviewed mutations of the $\alpha 1$, $\gamma 2$ and δ subunits of the GABA_A receptor that are associated with different idiopathic generalized epilepsy syndromes.

Mody and Pearce (2004) reviewed the diversity of inhibitory neurotransmission through GABA_A receptors.

Rudolph and Möhler (2004) reported analysis of GABA_A receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Whereas the knockout mice have provided information primarily with respect to the regulation of subunit gene transcription, receptor assembly, and some physiological functions of individual receptor subtypes, the point-mutated knockin mice in which specific GABA_A receptors are insensitive to diazepam or some general anesthetics have revealed the specific contribution of individual receptor subtypes to the pharmacological spectrum of diazepam and cerebral anesthetics.

Among GABA_A agonists and partial antagonists, Krosggaard-Larsen et al. (2004) characterized THIP (Gaboxadol) as a non-opioid analgesic and a novel type of hypnotic.

Boehm et al. (2004) described γ -aminobutyric acid receptor subunit mutant mice, which offer new perspectives on alcohol actions.

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- The receptor on which baclofen acts is coupled via G_i/G_o proteins to Ca²⁺ and K⁺ channels as well as adenylyl cyclase in neurons and hence is classified as a metabotropic receptor.
- As in other receptor families, heterogeneity of the GABA_B receptor has been found (Scherer et al. 1988; Bittiger et al. 1992; Bonanno and Raiteri 1992, 1993a, b; Bowery 1993; Lanza et al. 1993). At least 3 distinct subtypes have been identified:
- a) the postsynaptic receptor linked via a G-protein to a K⁺ channel which upon stimulation by GABA hyperpolarizes the neuron,
 - b) the presynaptic autoreceptor at GABA nerve endings; blockade of this receptor augments the release of GABA in electrically stimulated rat cortical slices,
 - c) the presynaptic heteroreceptor at glutamate nerve endings; blockade with GABA_B antagonists increases release of glutamate from K⁺ stimulated cortical slices.
- The structure of GABA_B receptors was identified when isoforms were detected (Kaupmann et al. 1997). Potent GABA_B antagonists were described (Bittiger et al. 1992, 1993; Froestl et al. 1996).

PROCEDURE

Materials

Radioligand: ³H-(–)baclofen, specific activity 1.11–1.85 TBq/mmol (30–50 Ci/mmol) New England Nuclear, Boston

Membrane Preparation

Rats are killed by decapitation, the cerebella quickly removed, and placed into ice-cold preparation buffer (50 mM Tris-HCl, pH 7.4). Approximately 5 g wet weight of the cerebella are homogenized using a glass Teflon potter, corresponding to 1 g cerebellum wet weight/50 ml buffer, and centrifuged at 48,000 g at 4°C for 10 min. The pellets are resuspended in approximately 270 ml preparation buffer, and centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebellum wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at –77°C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed, and centrifuged at 50,000 g for 20 min. The pellets are resuspended in the same volume of incubation buffer, and stirred for 45 min at room temperature. The

E.2.1.1.4

GABA_B Receptor Binding

PURPOSE AND RATIONALE

Baclofen, as an analogue of the inhibitory neurotransmitter γ -aminobutyric acid (GABA), binds as agonist to the subtype B of the GABA receptor. Baclofen is effective in the treatment of spasticity caused by multiple sclerosis or other diseases of the spinal cord, particularly traumatic lesions. Studies of similar compounds may lead to other effective antispasmodic drugs.

In rat cerebellar membranes, only the GABA_B receptor is labeled in the given concentration range of the GABA_B selective agonist ³H-(–)baclofen. The assay allows specifically the estimation of the test drug's binding characteristics at the GABA_B subtype receptor population.

suspension is recentrifuged as before. This washing step is repeated 3 times. The resulting pellets are re-suspended in ice-cold incubation buffer in a volume, yielding a membrane suspension with a protein content of 1 mg/ml. The membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Assay

For each concentration samples are used in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates). The concentration of ³H-(–)baclofen is constant in all samples ($1.8\text{--}2 \times 10^{-8}$ M).

Saturation Experiments

Total Binding

- 50 µl ³H-(–)baclofen
- 50 µl non-radioactive racemic baclofen (15 concentrations, $0\text{--}1.2 \times 10^{-6}$ M)

Non-Specific Binding

The measurement of the non-specific binding is performed at the lowest concentration of the saturation range, i. e. 1.8×10^{-8} M of the ³H-(–)baclofen without non-radioactive racemic baclofen.

- 50 µl ³H-(–)baclofen
- 50 µl gamma-aminobutyric acid.

Competition Experiments

- 50 µl ³H-(–)baclofen
- 50 µl incubation buffer without or with labeled test drug (15 concentrations, $10^{-10}\text{--}10^{-3}$ M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1 mg protein/1 ml). The samples are incubated for 60 min at 4°C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fiber filters. Thereby, the membrane bound is separated from the free radioactivity. The filters are washed with approximately 20 ml ice-cold buffer. The retained membrane bound radioactivity on the filter is measured after addition of 0.3 ml ethylene glycol monomethyl ether and 2 ml liquid scintillation cocktail per sample and an equilibration time of 1 h in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding – non-specific binding.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ³H-(–)baclofen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D {}^3\text{H} \times \text{IC}_{50}}{K_D {}^3\text{H} + [{}^3\text{H}]}$$

IC_{50} = concentration of the test drug, which inhibits 50% of specifically bound ³H-(–)baclofen in the competition experiment.

[³H] = concentration of ³H-(–)baclofen in the competition experiment

$K_D {}^3\text{H}$ = dissociation constant of ³H-(–)baclofen, determined from the saturation experiment.

The K_i -value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

MODIFICATIONS OF THE METHOD

Kaupmann et al. (1998) showed that GABA_B-receptor subtypes assemble into functional heteromeric complexes.

Bowery and Enna (2000) demonstrated that GABA_B receptors are functional metabotropic heterodimers.

Molecular structure and physiological functions of GABA_B receptors are reviewed by Bettler et al. (2003) and Bowery et al. (2003).

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E.2.1.2

Benzodiazepine Receptor: [³H]-Flunitrazepam Binding Assay

PURPOSE AND RATIONALE

Experiments using ³H-diazepam or ³H-flunitrazepam have demonstrated specific binding sites in CNS membrane preparations that satisfy the criteria for pharmacological receptors, e.g. saturability, reversibility, stereoselectivity and significant correlation with *in vivo* activities of the drugs in this class.

Heterogeneity of benzodiazepine receptors has been reported (Klepner et al. 1979; Supavilai and Karobath 1980; Hafely et al. 1993; Davies et al. 1994). There are four classes of benzodiazepine and non-benzodiazepine high affinity ligands for benzodiazepine recognition sites associated with GABA_A receptors: The first class (e.g. diazepam, flunitrazepam, alprazolam) facilitates the action of GABA, increasing the opening frequency of Cl[–] channels. These ligands are called full positive allosteric modulators, or full agonists. A second class of ligands, which includes the β -carbolines, can decrease the opening frequency of Cl[–] channels. These ligands are known as full negative allosteric modulators, or full inverse agonists. A third class (e.g. flumazenil) binds with high affinity to ben-

zodiazepine recognition sites, but it can also prevent the GABA modulations elicited by positive or negative allosteric modulators; this class is called a modulator antagonist. A fourth class of ligands for benzodiazepine recognition sites is known to elicit either partial amplification or partial attenuation of GABA action at various GABA_A receptors, and comprises the class called partial positive and partial negative allosteric modulators or partial agonists and partial inverse agonists, respectively.

The names ω_1 , ω_2 , and ω_3 -receptor subtypes have been proposed to replace the nomenclature of benzodiazepine BZ₁, BZ₂, and BZ_p receptors (Langer and Arbilla 1988; Langer et al. 1990; Griebel et al. 1999a, b).

PROCEDURE

Reagents

- [Methyl-³H]-Flunitrazepam (70–90 Ci/mmol) can be obtained from New England Nuclear.
- Clonazepam HCl can be obtained from Hoffmann La Roche

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. The cerebral cortices are removed, weighed and homogenized with a Potter-Elvehjem homogenizer in 20 volumes of ice-cold 0.32 M sucrose. This homogenate is centrifuged at 1000 *g* for 10 min. the pellet is discarded and the supernatant is centrifuged at 30,000 *g* for 20 min. The resulting membrane pellet is resuspended in 40 volumes of 0.05 M Tris buffer, pH 6.9.

Assay

- 1 ml 0.05 M Tris buffer, pH 6.9
- 560 μ l H₂O
- 70 μ l 0.5 M Tris buffer, pH 6.9
- 50 μ l ³H-Flunitrazepam
- 20 μ l vehicle (for total binding) or 0.1 mM Clonazepam (for non-specific binding) or appropriate drug concentrations.
- 300 μ l tissue suspension.

The tubes containing ³H-flunitrazepam, buffer, drugs and H₂O are incubated at 0–4°C in an ice bath.

A 300 μ l aliquot of the tissue suspension is added to the tubes at 10-s intervals. The timer is started with the addition of the mixture to the first tube. The tubes are then incubated at 0–4°C for 20 min and the assay stopped by vacuum filtration through Whatman GF/B filters. This step is performed at 10-s intervals. Each filter is immediately rinsed with three 5-ml washes of ice-cold buffer, pH 6.9. The filters are counted in 10 ml of liquid scintillation counting cocktail.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of clonazepam. Specific binding is approximately 97% of total ligand binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC₅₀ calculations are performed using log-probit analyses.

CRITICAL ASSESSMENT OF THE METHOD

Binding to the benzodiazepine receptor is not absolutely predictive for anxiolytic activity. A range of compounds have been discovered that do not have the benzodiazepine structure but that do interact with the benzodiazepine receptors (Gardner 1988; Byrnes et al. 1992). They may have a different pharmacological profile *in vivo*.

MODIFICATION OF THE METHOD

Takeuchi et al. (1992) developed a non-isotopic receptor assay for benzodiazepine drugs using the biotin-1012-S conjugate. The free conjugate in the supernatant was determined with a solid-phase avidin-biotin binding assay.

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E.2.1.3

Serotonin Receptor Binding

E.2.1.3.1

General Considerations

Several surveys on 5-HT receptors and their classification have been published, e.g., by Humphrey et al. (1993), Peroutka (1993), Boess and Martin (1994), Hoyer et al. (1994), Keabian and Neumeyer (1994), Martin and Humphrey (1994), Saxena (1994), Brancheck (1995), Sleight et al. (1995), Bockaert et al. (1997), Brancheck and Zgombick (1997), Briley et al. (1997), Costal and Naylor (1997), Glennon and Dukat (1997), Göthert and Schlicker (1997), Hamon (1997), Hartig (1997), Hoyer and Martin (1997), Jacobs and Fornal (1997), Roth and Hyde (1997), Uphouse (1997), Martin (1998), Martin and Eglen (1998), Saxena et al. (1998), Barnes and Sharp (1999), Pauwels (2000), and Gershon (2004).

The classification has evolved from a scheme recognizing three classes (5-HT_{1like}, 5-HT₂ and 5-HT₃) to one accepted by the NC-IUPHAR subcommittee for 5-hydroxytryptamine (serotonin) receptors in which seven classes embrace 14 distinct receptor subtypes (Martin 1988; Martin and Eglen 1998). Some revisions of the nomenclature were made:

Renaming the 5-HT_{1C} receptor to 5-HT_{2C} on the basis of recognitory, transductional and structural identity with the 5-HT₂ family.

Alignment of the classification scheme with the human genome, meaning that human receptors are given pre-eminence in the nomenclature.

Renaming the 5-HT_{1D α} and 5-HT_{1D β} subtypes to 5-HT_{1D} and 5-HT_{1B}, respectively.

Recognition that the '5-HT_{1like}' positively coupled to adenylate cyclase and mediating smooth muscle relaxation, is the 5-HT₇ receptor.

Use of a lower-case notation to describe a putative receptor defined only by gene product, with 'promotion' to an upper-case notation when the receptor is fully defined in terms of operational, recognitory and structural properties.

Up to 7 functional isoforms of the 5-HT_{2C} receptor, two functional isoforms of the 5-HT₄ receptor and four isoforms of the 5-HT₇ receptor were recognized.

Murphy et al. (1999) reviewed molecular biology-based alterations in 5-HT receptors including al-

tered characteristics of mice lacking different 5-HT receptors, e. g., 5-HT_{1B}-receptor-deficient mice, 5-HT_{2C} receptor-deficient mice, 5-HT_{1A}-receptor-deficient mice, 5-HT cell-membrane-transporter deficient mice and vesicular monoamine-transporter-deficient mice.

The role of 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2B}, and 5-HT₇ receptors in cardiovascular physiology and pharmacology was discussed by Watts and Cohen (1999).

5-HT Receptor Types and Subtypes

5-HT₁ Subtypes. At least five *5-HT₁ subtypes* are described (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}), which share 41–63% overall sequence identity and couple preferentially to G_{i/o} to inhibit cAMP formation.

Hartig et al. (1996) suggested a revised nomenclature for 5-HT_{1B}, 5-HT_{1D α} and 5-HT_{1D β} receptor subtypes.

5-HT_{1A} receptor agonists were described by Sills et al. (1984), Porsolt et al. (1992), Foreman et al. (1994), Wolff et al. (1997), Hamon (1997), 5-HT_{1A} receptor antagonists by Allen et al. (1997), 5-HT_{1B/D} receptor antagonists by de Vries et al. (1997), 5-HT_{1D} receptor agonists by Macor et al. (1994), van Lommen et al. (1995), Valentin et al. (1996), 5-HT_{1D} receptor antagonists by Clitherow et al. (1994), de Vries et al. (1996), Rollema et al. (1996), Briley et al. (1997).

Ramboz et al. (1998) recommended serotonin receptor 1A knockout mice as an animal model of anxiety-related disorder.

Presynaptic receptors may be preferably involved in the anxiolytic effects of 5-HT_{1A} receptor agonists, whereas in the antidepressant effects postsynaptic receptors are strongly involved (De Vry 1991, 1995).

5-HT₁ receptors are involved in learning and memory processes (see F.3.3.7 and F.3.4.1).

An endogenous peptide interacting specifically with the serotonergic 1B receptor subtypes was identified (Rousselle et al. 1996).

5-HT autoreceptors, mainly of the 5-HT_{1D} subtype, were studied by Starke et al. (1989), Fink et al. (1995), Bühlen et al. (1996), Glennon et al. (1996), Roberts et al. (1996), Price et al. (1996).

Cushing et al. (1994) studied the role of a 5-HT_{1D}-like receptor in serotonin-induced contraction of canine coronary artery and saphenous vein.

Wainscott et al. (2005) and Lucaites et al. (2005) described [³H]Y334370 as a radioligand for the 5-HT_{1F} receptor.

See E.2.1.3.2 and E.2.1.3.3 for binding assays of 5-HT₁ receptors.

5-HT₂ Receptors. *5-HT₂ receptors* have been subdivided into 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, which exhibit 46–50% overall sequence identity and couple preferentially to G_{q/11} to increase the hydrolysis of inositol phosphates and elevate cytosolic [Ca²⁺]. (Humphrey et al. 1993; Hoyer et al. 1994; Kebebian and Neumeyer 1994; Martin and Humphrey 1994; Saxena 1994; Shih et al. 1994; Tricklebank 1996; Martin 1998). The human 5-HT₂ receptors were cloned and characterized (Chen et al. 1992; Carey et al. 1996). Species differences in receptors were described (Johnson et al. 1995). 5-HT₂ receptors play a role in the action of antipsychotics and hallucinogens.

Saucler and Albert (1997) identified an endogenous 5-hydroxytryptamine_{2A} receptor in NIH-3T3 cells.

Wolf and Schutz (1997) found that the serotonin 5-HT_{2C} receptor is a prominent serotonin receptor in basal ganglia.

Bonhaus et al. (1995) described the pharmacology and distribution of human 5-hydroxytryptamine_{2B} (5-HT_{2B}) receptor gene products and compared this with 5-HT_{2A} and 5-HT_{2C} receptors.

Chagraoui et al. (2003) reported that agomelatine (S20088) antagonizes the penile erections induced by the stimulation of 5-HT_{2C} receptors in Wistar rats.

See E.5.1.9. and E.5.1.10 for binding assays to 5-HT₂ receptors.

5-HT₃ Receptor. The *5-HT₃ receptor* is a pentameric, ligand-gated ion channel, activation of which promotes entry of Na⁺ and Ca²⁺, egress of K⁺ and hence neuronal depolarization. It belongs to the transmitter-gated cation super-family of receptors and appears to be located exclusively in neuronal tissue where it mediates fast depolarization (Malone et al. 1991). Responses are blocked by a wide range of potent antagonists, which are highly selective with respect to other 5-HT receptors (Silverstone and Greenshaw 1996). The Bezold-Jarisch reflex can be elicited by 5-HT₃ receptor agonists and be blocked by 5-HT₃ antagonists (see A.1.3.19). 5-HT₃ receptor antagonists are used as anti-migraine drugs. 5-HT₃ receptors are involved in feeding behavior (see L.3.1.1) Furthermore, gastric emptying (see J4.4.2) and emesis (see J.5.0.1, J.5.0.2, J.5.0.3 and J.5.0.4) can be influenced by 5-HT₃ antagonists.

5-HT₃ receptor antagonists, such as **ondansetron** (Koulu et al. 1990; Ishizuka et al. 1993; Miyata et al. 1995; Cappelli et al. 1996), **granisetron** (Sanger and Nelson 1989; Ito et al. 1995; Endo et al. 1999; Yan and White 2005), **tropisetron** (Macor et al. 2001; de la

Vega et al. 2005), **zacopride** (Waeber et al. 1990; Kidd et al. 1992), and **alosetron** (Clayton et al. 1999) are used clinically in the prevention of cisplatin-induced emesis (Karim et al. 1996; Roila et al. 1997).

The synthesis and pharmacological profile of several selective 5-HT₃ receptor antagonists have been reported (Fitzpatrick et al. 1990; Rosen et al. 1990; Swain et al. 1991; Ito et al. 1992; Bachy et al. 1993; Heidempergher et al. 1997; Rival et al. 1998; Modica et al. 2004).

Boess et al. (1997) analyzed the ligand binding site of the 5-KT₃ receptor using site direction mutagenesis.

See E.2.1.3.4 for binding assays to the 5-HT₃ receptor.

5-HT₄ Receptors. The 5-HT₄ receptors couple preferentially to G_S protein and activate adenylate cyclase, thereby increasing intracellular cAMP levels (Martin 1998). This induces long-term modulation of ion-channel activity, which is fundamental in learning and memory (Eglen et al. 1995; Eglen and Hedge 1996). Excitatory responses were found in guinea pig ileum and colon, inhibitory responses in rat esophagus (Ford and Clarke 1993).

The tunica muscularis mucosae preparation of the rat esophagus has been recommended for evaluation of 5-HT₄ receptor ligands since it possesses a homogeneous population of 5-HT₄ receptors which mediates a well defined relaxant response to 5-HT (see J.2.0.1)

5-HT₄ receptors may mediate arrhythmias (Kauermann 1994). Two splice variants have been identified (Gerald et al. 1995). Several selective 5-HT₄ receptors agonists and antagonists were described (Gaster et al. 1995; Eglen and Hedge 1996; Eglen 1997).

Dumuis et al. (1992) characterized DAU 6285, a novel 5-HT₄ receptor antagonist of the azabicycloalkyl benzamidozolone class.

Wardle et al. (1994) described the effects of SB 204070, a highly potent and selective 5-HT₄ receptor antagonist on guinea pig distal colon.

The 5-HT₄ binding site in human brain was characterized by Arranz et al. (1998).

López-Rodríguez et al. (1999) reported the synthesis and structure–activity relationships of new benzimidazole-4-carboxamides and carboxylates as potent and selective 5-HT₄ receptor antagonists.

Radioligand binding assays for 5-HT₄ receptors using [³H]-GR113808 have been described by Grossman et al. (1993), Domenech et al. (1994), Schiavi et al. (1994), Katayama et al. (1995), Ansanay et al. (1996).

Brattelid et al. (2004) reported cloning, the pharmacological characterization and tissue distribution of a novel 5-HT₄ receptor splice variant, 5-HT_{4i}.

A survey on molecular biology and potential functional role of **5-HT₅, 5-HT₆, and 5-HT₇ receptors** was given by Branchek and Zoombick (1997).

A review on 5-HT₅ receptors was given by Nelson (2004).

5-HT₆. The rat 5-HT₆ receptor has been cloned by Ruat et al. (1993). The distinguishing features of this receptor are the high affinity for a series of antipsychotic compounds as well as affinity for a number of cyclic antidepressants (Branchek 1995).

Bourson et al. (1995) used antisense oligonucleotides to determine the role of the 5-HT₆ receptor in the rat brain.

Boess et al. (1998) reported labelling of 5-hydroxytryptamine binding sites in rat and porcine striatum by the 5-HT₆ receptor-selective ligand [³H]Ro 63–0563.

Branchek and Blackburn (2000) reviewed 5-HT₆ receptors as emerging targets for drug discovery.

A review on 5-HT₆ receptors was given by Wooley et al. (2004).

Routledge et al. (2000) characterized SB-271046 as a potent, selective and orally active 5-HT₆ receptor antagonist.

Wooley et al. (2003) reported the reversal of cholinergic-induced deficit in a rodent model of recognition memory by the selective 5-HT₆ receptor antagonist Ro 04–6790.

5-HT₇. Lovenberg et al. (1993), Shen et al. (1993), Gobbi et al. (1996), Villalón et al. (1997) described an adenylyl cyclase-activating serotonin receptor (5-HT₇) implicated in the regulation of mammalian circadian rhythms.

A receptor autoradiographic and hybridization analysis of the distribution of the 5-HT₇ receptor in rat brain was reported by Gustafson et al. (1996).

Stowe and Barnes (1998) used [³H]5⁻carboxamidotryptamine for selective recognition sites in rat brain.

Three different splice variants of the 5-HT₇ receptor have been described both in rat and human tissues (Vanhoenacker et al. 2000).

Roth et al. (1994) described the binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors.

Hedlund and Sutcliffe (2004) reviewed the functional, molecular, and pharmacological advances in 5-HT₇ receptor research.

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E.2.1.3.2

Serotonin (5-HT_{1A}) Receptor: Binding of [³H]-8-Hydroxy-2-(di-n-Propylamino)-Tetralin ([³H]-DPAT)

PURPOSE AND RATIONALE

Determination of the affinity of test compounds for the 5-HT_{1A} receptor in brain may be useful for predicting compounds with novel anxiolytic or atypical anti-psychotic profiles.

The existence of at least two populations of 5-HT₁ receptors in rat brain was shown by differential sensitivity to spiroperidol (Pedigo et al. 1981). The spiroperidol-sensitive receptors were designated

as the 5-HT_{1A} subtype and the insensitive receptors were referred to as the 5-HT_{1B} subtype (Middlemiss and Fozard 1983). Other 5-HT binding sites (5-HT_{1C}, 5-HT_{1D}, 5-HT₃ and 5-HT₄) have subsequently been identified in various species, based on differential sensitivity to 5-HT antagonists (Peroutka 1988).

Schlegel and Peroutka (1986) identified [³H]DPAT as a selective ligand for the 5-HT_{1A} receptor. These authors reported that [³H]DPAT labeled an autoreceptor. Lesion studies suggest that [³H]DPAT labeled receptors are not terminal autoreceptors, but may be somatodendritic autoreceptors (Gozlan et al. 1983). Although DPAT decreases the firing rate in the raphe nucleus and inhibits 5-HT release, the actual location and function is somewhat controversial (Verge et al. 1986). These studies and the sensitivity of [³H]DPAT binding to guanine nucleotides and effects on adenylate cyclase suggest that DPAT acts as an agonist at the 5-HT_{1A} receptor (Schlegel and Peroutka 1986).

Serotonin may play a role in anxiety, since drugs which reduce serotonergic function have anxiolytic effects in animal models (Dourish et al. 1986). Since buspirone and its analogs have relatively higher affinity for the 5-HT_{1A} receptor than other receptors and no effect on the benzodiazepine site, their anxiolytic properties are attributed to activity at the 5-HT_{1A} receptor (Verge et al. 1986; Iversen 1984; Traber and Glaser 1987).

Besides 5-HT_{1A} receptor agonists (Misslin et al. 1990; Griebel et al. 1992; Hascoet et al. 1994; Stanhope and Dourish 1996), 5-HT_{1A} receptor antagonists (Traber and Glaser 1987; Fletcher et al. 1996; Johansson et al. 1997; Cao and Rodgers 1998), 5-HT_{2A} receptor antagonists (Griebel 1996), 5-HT_{2C} receptor antagonists (Jenck et al. 1998), mixed receptor agonists/antagonists (Kleven et al. (1997), 5-HT₃ receptor antagonists (Artais et al. 1995; Roca et al. 1995) and 5-HT₄ receptor antagonists (Kennett et al. 1997) exhibit anxiolytic properties (Handley and McBlane 1993).

Fletcher et al. (1995) described visualization and characterization of 5-HT receptors and transporters *in vivo* and in man.

Several 5-HT_{1A} receptor antagonists were described in preclinical studies, such as **NAN-190** (Deans et al. 1989; Rydelek-Fitzgerald et al. 1990; Neckelmann et al. 1996), **(-)-alprenolol** (Bjorvatn et al. 1992), **pindolol** (Bel et al. 1994; Dreshfield et al. 1996; Seifritz et al. (1997), **BMY 7378** (Sharp et al. 1990; Grasby et al. 1992), **WAY 100635** (Fletcher et al. 1996; Trillat et al. 1998; Harder and Ridley 2000; Smart and Biello 2001), **(-)-tertatolol** (Jolas et al. 2004).

Millan et al. (1999, 2001), Mattson et al. (2003), and Gilbert et al. (2004) described specific 5-HT_{1A} receptor antagonists.

PROCEDURE

Reagents

- Tris buffers, pH 7.7
 - 57.2 g Tris HCl
16.2 g Tris base
q.s. to 1 liter with distilled water (0.5 M Tris buffer, pH 7.7)
 - Make a 1:10 dilution in deionized H₂O (0.05 M Tris buffer, pH 7.7)
 - 0.05 M Tris buffer, pH 7.7 containing 10 μM pargyline, 4 mM CaCl₂ and 0.1% ascorbic acid.
0.49 mg pargyline HCl
111 mg CaCl₂
250 mg ascorbic acid
q.s. to 250 ml with 0.05 M Tris buffer, pH 7.7 (reagent 1b)
- [³H]-DPAT (2-(N,N-Di[2,3(n)-³H]propylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene) (160–206 Ci/mmol) was obtained from Amersham. For IC₅₀ determinations: a 10 nM stock solution is made up and 50 μl are added to each tube (final concentration = 0.5 nM).
- Serotonin creatinine sulfate. 0.5 mM stock solution is made up in 0.01 N HCl and 20 μl added to 3 tubes for determination of nonspecific binding (final concentration = 10 μM).
4. Test compounds:
For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 2 × 10⁻⁵ to 2 × 10⁻⁸ M. Seven concentrations are used for each assay. Higher or lower concentrations may be used based on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Hippocampi are removed, weighed and homogenized in 20 volumes of 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 g for 10 min and the supernatant is discarded. The pellet is resuspended in an equal volume of 0.05 M Tris buffer, incubated at 37°C for 10 min and recentrifuged at 48,000 g for 10 min. The final membrane pellet is resuspended in 0.05 M Tris buffer containing 4 mM CaCl₂, 0.1% ascorbic acid and 10 μM pargyline.

Assay

800 μl Tissue

130 µl 0.05 M Tris + CaCl₂ + pargyline + ascorbic acid

20 µl vehicle/5-HT/drug

50 µl [³H]DPAT

Tubes are incubated for 15 min at 25°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 2 times with 5 ml of ice-cold 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml of Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 10 µM 5-HT. IC₅₀ values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng–Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D$$

The K_D value for [³H] DPAT binding was found to be 1.3 nM by Scatchard analysis in a receptor saturation experiment.

MODIFICATIONS OF THE METHOD

Yocca et al. (1987) described BMY 7378, a buspirone analog with high affinity, selectivity and low intrinsic activity at the 5-HT_{1A} receptor in rat and guinea pig hippocampal membranes.

Instead of the selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (DPAT), selective antagonists to the 5-HT_{1A} receptor, 4-(methoxyphenyl)-1-[2'-(n-2''-pyridinyl)-p-iodobenzamido]-ethyl-piperazine ([¹²⁵I]p-MPPI), and [³H]p-MPPF = 4-(2'-methoxy-)-phenyl-1-[2'-(N-2''-pyridyl)-p-fluorobenzamido] ethyl-piperazine have been recommended (Kung et al. 1994a, 1994b, 1995, 1996).

Several other selective 5-HT_{1A} receptor radioligands were recommended:

[³H]lisuride (Sundaram et al. 1995); [³H]WAY-100635 ([O-methyl-³H]-N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl)cyclohexane carboxamide trihydrochloride) (Laporte et al. 1994; Gozlan et al. 1995; Hume et al. 1995; Khawaja 1995; Khawaja et al. 1997); [³H]Alnespirone (Fabre et al. 1997); [Carbonyl¹¹C]-Desmethyl-WAY 100635 (Pike et al. 1998); [³H]S 15535 (4-(benzodioxan-5-yl)-1-(indan-2-yl)piperazine) (Newman-Tancredi et al. 1998a); NAD-299 (= (R)-3-N,N-dicyclobutylamino-8-fluoro-

[6-³H]-3,4-dihydro-2H-1-benzopyran-carboxamide) (Jerning et al. 1998; Sandell et al. 1999).

Newman-Tancredi et al. (1998b) performed autoradiographic studies with [³⁵S]-GTPγS and the selective radioligand [³H]S 15535 for parallel evaluation of localization and functionality of the 5-HT_{1A} receptor.

Agonist-Stimulated [³⁵S]-GTPγS Binding

Agonist-stimulated [³⁵S]-GTPγS binding is the initial step in signal transduction following G protein activation (Elmendorf et al. 1998; Standaert et al. 1998; Lee et al. 1999). It can be used as an index of receptor stimulation, such as 5-HT_{1A} receptors (Alper and Nelson 1998, 2000; Hughes et al. 2005).

PROCEDURE

Sprague Dawley rats were killed by decapitation, the brains placed in ice-cold 0.9% NaCl for 1–3 min, the hippocampi dissected free-hand and placed in a vial on ice. The tissue was weighed and frozen at -70°C. Upon defrosting the tissue was homogenized using a Tekmar tissue homogenizer in cold Tris buffer (50 mM Tris base, pH 7.4). The homogenate was centrifuged (39,800 g, 4°C for 10 min), the supernatant decanted and the remaining pellet resuspended in the same Tris buffer. After homogenization the suspension was incubated at 37°C for 10 min in a shaking water bath. The suspension was then centrifuged, the supernatant decanted and the pellet again washed with cold Tris buffer. After one final centrifugation, the remaining pellet was homogenized in the same Tris buffer at a final concentration of approximately 100 mg tissue/ml. This suspension was frozen in aliquots at -70°C for later use. On the day of the assay, the frozen tissue suspension was defrosted, resuspended in approximately 35 ml Tris buffer and centrifuged as above. This final membrane pellet was suspended in assay buffer (4 mM MgCl₂, 160 mM NaCl, 0.267 mM EGTA, 67 mM Tris base, pH 7.4) to produce a protein concentration approximating 200 µg/ml.

GTPγS Binding Assay

To each assay tube was added 200 µl deionized water or drug, 200 µl [³⁵S]GTPγS (0.1 nM) in ligand buffer (assay buffer containing 1200 µM GDP to produce a final concentration of 300 µM), 200 µl assay buffer and 200 µl tissue homogenate (approximately 30–50 µg protein). The assay tubes were incubated for 30 min in a shaking water bath. All assays were conducted at 37°C. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters pre-

wet with 20 mM Na₄P₂O₇·10H₂O and pre-cooled with three washes of 2 ml cold 50 mM Tris buffer. After filtering the reaction mixture, the filters were washed 4 times with 1 ml cold 50 mM Tris buffer, placed into 7-ml scintillation vials and 5 ml Ready Protein scintillation cocktail added. The samples were shaken, allowed to sit for a minimum of 2 h, shaken again and counted (2 min/sample) in a Beckman scintillation counter. Non-specific binding was determined by the amount of [³⁵S]-GTPγS bound in the presence of 10 μM unlabeled GTPγS and was subtracted from all samples. Basal GTPγS binding is defined as the specific binding when 200 μl water containing no ligand was added to the assay tube. This is also referred to as agonist-independent binding.

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E.2.1.3.3

Serotonin (5-HT_{1B}) Receptors in Brain: Binding of [³H]5-Hydroxytryptamine ([³H]5-HT)

PURPOSE AND RATIONALE

The purpose of this assay is to determine the affinity of test compounds for the serotonin (5-HT_{1B}) receptor in brain.

The existence of two populations of 5-HT₁ receptors in rat brain was shown by differential sensitivity to spiroperidol (Pedigo et al. 1981). The spiroperidol-sensitive receptors were designated as the 5-HT_{1A} subtype and the insensitive receptors were referred to as the 5-HT_{1B} subtype (Middlemiss and Fozard 1983). The 5-HT_{1B} subtype has been identified in the brain of rats and mice (Peroutka 1986) and can be selectively labeled by 5-HT in rat striatum when spiroperidol is included to mask the 5-HT_{1A} and 5-HT₂ receptors. In contrast to the situation in rats and mice,

[³H]5-HT binding in the basal ganglia of other mammals displays a pharmacological profile characteristic of 5-HT_{1D} sites. The distribution of 5-HT_{1B} sites in rat brain is similar to that of 5-HT_{1D} sites in human brain (Segu et al. 1991; Boulenguez et al. 1992; Palacios et al. 1992). Several 5-HT_{1B} antagonists have been described (Price et al. 1997; Selkirk et al. 1998).

By comparing the results in the 5-HT_{1B} assay with those in the 5-HT_{1A}, 5-HT₂ and the 5-HT₃ receptor binding assays the relative affinity of a test compound for the major subclasses of 5-HT receptors in the rat brain can be determined.

PROCEDURE

Reagents

- Tris buffers, pH 7.7
 - 57.2 g Tris HCl
16.2 g Tris base
q.s. to 1 liter with distilled water (0.5 M Tris buffer, pH 7.7)
 - Make a 1:10 dilution in deionized H₂O (0.05 M Tris buffer, pH 7.7)
 - 0.05 M Tris buffer, pH 7.7 containing 10 mM pargyline, 4 mM CaCl₂ and 0.1% ascorbic acid.
0.49 mg pargyline HCl
110.99 mg CaCl₂
250 mg ascorbic acid
q.s. to 250 ml with 0.5 M Tris buffer, pH 7.7.
- 5-Hydroxy[G-³H]tryptamine creatinine sulfate (17–20 Ci/mmol) (Amersham). For IC₅₀ determinations: a 40 nM stock solution is made up and 50 μl added to each tube (final concentration = 2.0 nM).
- Serotonin creatinine sulfate. A 0.5 mM stock solution is made up in 0.01 N HCl and 20 ml added to 3 tubes for determination of nonspecific binding (final concentration = 10 μM).
- Spiroperidol is dissolved in dilute glacial acetic acid. A 20 μM stock solution is prepared and 50 μl added to each tube to prevent binding to 5-HT_{1A} and 5-HT₂ receptors (final concentration in the assay = 1 μM).
- Test compounds:
For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2 × 10⁻⁵ to 2 × 10⁻⁸ M. Seven concentrations are used for each assay. Higher or lower concentrations may be used based on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed and homogenized in 20 vol-

umes of 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 g for 10 min and the supernatant is discarded. The pellet is resuspended in an equal volume of 0.05 M Tris buffer, incubated at 37°C for 10 min and recentrifuged at 48,000 g for 10 min. The final membrane pellet is resuspended in 0.05 M Tris buffer containing 4 mM CaCl₂, 0.1% ascorbic acid and 10 mM pargyline.

Assay

| | |
|--------|-------------------------------------------------------------|
| 800 μl | tissue |
| 80 μl | 0.05 M Tris + CaCl ₂ + pargyline + ascorbic acid |
| 20 μl | vehicle/ 5-HT/ drug |
| 50 μl | [³ H]5-HT |
| 50 μl | spiroperidol |

Tubes are incubated for 15 min at 25°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 2 times with 5 ml of ice-cold 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml of Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 10 μM 5-HT. IC₅₀ values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng-Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D$$

The K_D value for [³H] 5-HT binding was found to be 16.5 nM by Scatchard analysis of a receptor saturation experiment.

MODIFICATIONS OF THE METHOD

[³H]CP-96,501 = 3-(1,2,5,6-Tetrahydro-4-pyridyl-5-n-propoxyindole was recommended as a selective 5-HT_{1B} receptor radioligand (Koe et al. 1992; Lebel and Koe 1992).

Domenech et al. (1997) characterized human serotonin 1D and 1B receptors using [³H]-GR-125743, a novel radiolabelled serotonin 5-HT_{1D/1B} receptor antagonist.

[³H]mesulergine has been used as radioligand for 5-HT_{1C} receptor binding (Jenck et al. 1993, 1994).

[³H]-5-carboxytryptamine was recommended as label for 5-HT_{1D} binding sites (Mahle et al. 1991; Novak et al. 1993).

Massot et al. (1998) described molecular, cellular and physiological characteristics of 5-HT-moduline, a tetrapeptide (Leu-Ser-Ala-Leu), acting as endogenous modulator of the 5-HT_{1B} receptor subtype.

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E.2.1.3.4

5-HT₃ Receptor in Rat Entorhinal Cortex Membranes: Binding of [³H]GR 65630

PURPOSE AND RATIONALE

The purpose of this assay is to determine the affinity of test compounds for the 5-HT₃ binding site in the brain. This assay may be useful for predicting the potential of compounds to exhibit anti-emetic, anxiolytic or atypical antipsychotic profiles.

The 5-HT₃ binding site has also been characterized on operational, transductional and structural characteristics. Originally it was believed that 5-HT₃ binding sites existed only in the periphery (Costall et al. 1988). However, with the introduction of potent and selective 5-HT₃ antagonists such as GR65630, zacopride, ICS 205 930 and MDL 72222, and agonists, data from binding studies have indicated that 5-HT₃ binding sites are also located in selected areas of the brain (Kilpatrick et al. 1987, 1989; Barnes et al. 1988, 1990, 1992; Watling et al. 1988; Miller et al. 1992).

The highest levels of 5-HT₃ binding sites have been detected in limbic and dopamine containing brain areas (entorhinal cortex, amygdala, nucleus accumbens and tuberculum olfactorium) (Costall et al. 1988). Besides possessing selective binding in dopamine rich areas, 5-HT₃ antagonists have been reported to block behavioral effects associated with certain drugs of abuse (nicotine and morphine) and to be active in behavioral tests predictive of anxiolytic activity. Based on these selective regional binding results and behavioral studies, it has been speculated that 5-HT₃ antagonists may have a therapeutic benefit in disease states believed to be associated with excessive dopaminergic activity; i. e., schizophrenia, anxiety and drug abuse.

Several authors described synthesis, pharmacology and therapeutic potential of H₃ receptor agonists and antagonists (Leurs et al. 1995, 1998; Stark et al. 1996).

PROCEDURE

Reagents

- 0.05 M Krebs-HEPES buffer, pH 7.4
11.92 g HEPES
10.52 g NaCl
0.373 g KCl
0.277 g CaCl₂
0.244 g MgCl₂·6H₂O
q.s. to 1 liter with distilled H₂O bring pH up to 7.4 (at 4°C) with 5N NaOH
- [³H]GR65630 (87.0 Ci/mmol) is obtained from New England Nuclear. For IC₅₀ determinations: [³H]GR65630 is made up to a concentration of 1.0 nM in Krebs-HEPES buffer such that when 100 μl is added to each tube a final concentration of 0.4 nM is attained in the 250 μl assay.
- Ondansetron HCl (GR 38032F) is made up to a concentration of 500 μM in Krebs-HEPES buffer. 50 μl is added to each of 3 tubes for the determination of nonspecific binding (yielding a final concentration of 100 μM in the 250 μl assay).
- Test compounds. For most assays, a 50 μM stock solution is made up in a suitable solvent and serially diluted with Krebs-HEPES buffer such that when 50 μl of drug is combined with the total 250 μl assay, a final concentration from 10⁻⁵ to 10⁻⁸ M is attained. Characteristically seven concentrations are studied for each assay; however, higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats (150–200 g) are decapitated, the entorhinal cortex removed, weighed and homogenized in

10 volumes of ice-cold 0.05 M Krebs-HEPES buffer, pH 7.4. The homogenate is centrifuged at 48,000 g for 15 min at 4°C. The resulting pellet is rehomogenized in fresh Krebs-HEPES buffer and recentrifuged at 48,000 g for 15 min at 4°C. The final pellet is resuspended in the original volume of ice-cold Krebs-HEPES buffer. This yields a final tissue concentration of 1.2–1.6 mg/ml with the addition of 100 ml to the assay. Specific binding is approximately 55–65% of total bound ligand.

Assay

- 100 μl Tissue suspension
100 μl [³H]GR65630
50 μl Vehicle (for total binding) or 500 μM ondansetron HCl (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept on ice for additions, then vortexed and incubated with continuous shaking for 30 min at 37°C. At the end of the incubation period, the incubate is diluted with 5 ml of ice-cold Krebs-HEPES buffer and immediately vacuum filtered through Whatman GF/B filters, followed by two 5-ml washes with ice-cold Krebs-HEPES buffer. The filters are dried and counted in 10 ml of liquid scintillation cocktail.

EVALUATION

Specific GR65630 binding is defined as the difference between the total binding and that bound in the presence of 100 μM Ondansetron HCl. IC₅₀ calculations are performed using computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dunn et al. (1991) found that preclinical data with 5-HT₃ antagonists predict anxiolytic rather than antipsychotic activity.

Davies et al. (1999) found that the 5-HT_{3B} subunit is a major determinant of serotonin receptor function.

Reiser and Hamprecht (1989) reported that substance P and serotonin (via 5-HT₃ receptors) act synergistically to activate the cation permeability as measured by [¹⁴C]guanidinium uptake in neuroblastoma × glioma hybrid cells.

Bönisch et al. (1993) studied the 5-HT₃ receptor-mediated cation influx into N1E-115 mouse neuroblastoma cells by the use of the organic cation [¹⁴C]-guanidinium.

Emerit et al. (1993) assessed the [¹⁴C]guanidium accumulation in cells of the hybridoma (mouse neuroblastoma × rat glioma) clone NG 108–15 exposed to serotonin 5-HT₃ receptor ligands and substance P.

Bonhaus et al. (1993) characterized the binding of [³H]endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazolone-1-carboxamide hydrochloride ([³H]BIMU-1), a benzimidazolone with high affinity to 5-HT₃ and 5-HT₄ receptors in NG 108 cells and guinea pig hippocampus.

Kooyman et al. (1994) studied the specific binding of [³H]GR65630 to 5-HT₃ recognition sites in cultured N1E-115 mouse neuroblastoma cells.

Several other selective 5-HT₃ receptor radioligands were recommended:

[³H]Quipazine (Perry 1990); [³H]GR67330 (Kilpatrick et al. 1990); tritium-labeled 1-methyl-N-[8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-1H-indazole-3-carboxamide (Robertson et al. 1990); [¹²⁵I]-(S)-iodozacopride (Gehlert et al. 1993); [³H]-BRL46470 (Steward et al. 1995); [³H]YM060 (Azukawa et al. 1995); (¹²⁵I)iodophenpropit (Jansen et al. 1994), (¹²⁵I)iodoproxyfan (Ligenau et al. 1994), (S)-Des-4-amino-3-[¹²⁵I]iodozacopride (Mason et al. 1996; Hewlett et al. 1998,1999).

Tairi et al. (1998) and Hovius et al. (1999) studied ligand binding to the serotonin 5-HT₃ receptor with a novel fluorescence ligand and developed a total internal reflection fluorescence assay which is suitable for high-through-put screening.

Thompson et al. (2005) reported locating an antagonist in the 5-HT₃ receptor binding site using modeling and radioligand binding.

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E.2.1.4

Histamine H₃ Receptor Binding in Brain

PURPOSE AND RATIONALE

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H₁ receptor couples mainly to G_{q/11}, thereby stimulating phospholipase C, whereas the H₂ receptor interacts with G_s to activate adenylyl cyclase. The histamine H₃ and H₄ receptors couple to G_i proteins to inhibit adenylyl cyclase, and to stimulate MAPK. The histamine H₃ receptor is regarded as a therapeutic target for cognitive and sleep disorders (Leurs et al. 1991, 1998, 2005; Passani et al. 2004).

Histamine modulates its own synthesis and release from depolarized brain slices or synaptosomes by interacting with H₃ autoreceptors with a pharmacology distinct from that of H₁ and H₂ receptors (Arrang et al. 1985, 1987, 1990; Hill 1990, 1992; Hill et al. 1997; Leurs et al. 1991, 1998). The R-isomer of α -methylhistamine (α -MeHA) was identified as a highly selective H₃-receptor agonist active at nanomolar concentrations. Furthermore, this compound in ³H-labeled form is a suitable probe for the H₃-receptor.

PROCEDURE

The cerebral cortex from guinea pigs is dissected and homogenized in 50 volumes ice-cold 50 mM KH₂PO₄/Na₂HPO₄ buffer, pH 7.5, in a Potter homogenizer. The homogenate is centrifuged for 15 min at 750 g. The pellet is discarded and the supernatant centrifuged at 42,000 g for 15 min. The supernatant is discarded and the pellet washed superficially with and then resuspended in fresh buffer. The protein concentration of the membrane suspension as determined according to Lowry et al. (1951) is about 0.3–0.4 mg/ml.

Aliquots of the membrane preparation are incubated for 60 min at 25°C with ³H(R) α -MeHA and unlabeled substances in a final volume of 1 ml. The assay is stopped by dilution with 2 × 3 ml ice-cold medium, followed by rapid filtration under vacuum over Millipore AAWP filters which are then rinsed twice with 5 ml of ice-cold medium. Radioactivity retained on the filters is measured by liquid scintillation spectroscopy.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of $10\mu\text{M}$ unlabeled $\alpha\text{-MeHA}$. IC_{50} values are calculated from the percent specific binding at each drug concentration. The K_i value may then be calculated by the Cheng-Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D$$

MODIFICATIONS OF THE METHOD

Jansen et al. (1992) described [^{125}I]iodophenpropit as a radiolabeled histamine H_3 receptor antagonist.

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E.2.2

Anticonvulsant Activity

E.2.2.1

Pentylentetrazole (Metrazol) Induced Convulsions

PURPOSE AND RATIONALE

This assay has been used primarily to evaluate anti-epileptic drugs. However, it has been shown that most anxiolytic agents are also able to prevent or antagonize Metrazol-induced convulsions.

PROCEDURE

Mice of either sex with a body weight between 18 and 22 g are used. The test compound or the reference drug is injected sc. or i.p. or given orally to groups of 10 mice. Another group of 10 mice serves as control. Fifteen min after sc.-injection, 30 min after i.p.-injection, or 60 min after oral administration 60 mg/kg MTZ (Metrazol) are injected subcutaneously. Each animal is placed into an individual plastic cage for observation lasting 1 h. Seizures and tonic-clonic convulsions are recorded. At least 80% of the animals in the control group have to show convulsions.

EVALUATION

The number of protected animals in the treated groups is calculated as percentage of affected animals in the control group. ED_{50} -values can be calculated. Furthermore, the time interval between MTZ-injection and occurrence of seizures can be measured. The delay of onset is calculated in comparison with the control group.

CRITICAL ASSESSMENT OF THE METHOD

The method is widely accepted as a screening procedure and has been modified by many investigators. Chlordiazepoxide (20 mg/kg i.p.), diphenylhydantoin

(10 mg/kg i.p.) and phenobarbitone sodium (20 mg/kg i.p.) were found to be effective. Predominantly, the muscle relaxant and anticonvulsant effects of benzodiazepines are measured by this test. Stimulant, anti-depressant, neuroleptic and some anti-epileptic drugs do not show MTZ-antagonism at tolerable doses (Lippa et al. 1979). Nevertheless, the antagonism of MTZ-induced seizures appears to be a suitable procedure for detecting compounds with potential anxiolytic activity. Among a battery of tests, the MTZ-antagonism has been proposed to study centrally acting skeletal muscle relaxants (Bastian et al. 1959; Domino 1964).

MODIFICATIONS OF THE METHOD

Different routes of administration (i.p., i.v., s.c.) have been used by various investigators. Moreover, the dose of MTZ which causes seizures in 80 to 90% of the animals varies with the strain being used.

Bastian et al. (1959) published a modification, whereby mice are infused with a MTZ-solution through a small-diameter polyethylene tubing into the tail vein. In the same animal 3 end-points are registered: 1. Onset of persistent clonic convulsions, 2. beginning of the tonic flexor phase, and 3. time to death. The three endpoints are affected differently by various drugs providing the basis for the determination of drug specificity.

Löscher et al. (1991) tested 8 clinically established antiepileptic drugs in three pentylentetrazole seizure models: (1) the threshold for different types of pentylentetrazole seizures, i.e., initial myoclonus twitch, generalized clonus with loss of righting reflexes and tonic backward extension of forelimbs (forelimb tonus) in mice; (2) the traditional pentylentetrazole seizure test with s.c. injection of the CD97 for generalized clonic seizures in mice; and (3) the s.c. pentylentetrazole seizure test in rats. Various factors may cause misleading predictions from pentylentetrazole seizure models.

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E.2.2.2

Strychnine-Induced Convulsions

PURPOSE AND RATIONALE

The convulsing action of strychnine is due to interference with postsynaptic inhibition mediated by glycine. Glycine is an important inhibitory transmitter to motoneurons and interneurons in the spinal cord, and strychnine acts as a selective, competitive antagonist to block the inhibitory effects of glycine at all glycine receptors. Strychnine-sensitive postsynaptic inhibition in higher centers of the CNS is also mediated by glycine. Compounds which reverse the action of strychnine have been shown to have anxiolytic properties.

PROCEDURE

Groups of 10 mice of either sex with a weight between 18 and 22 g are used. They are treated orally with the test compound or the standard (e.g. diazepam 5 mg/kg). One hour later the mice are injected with 2 mg/kg strychnine nitrate i.p. The time until occurrence of tonic extensor convulsions and death is noted during a 1 h period. With this dose of strychnine convulsions are observed in 80% of the controls.

EVALUATION

ED_{50} -values are calculated using various doses taking the percentage of the controls as 100%. For time-response curves the interval between treatment and strychnine injection varies from 30 to 120 min.

CRITICAL ASSESSMENT OF THE METHOD

The method has been proven to be useful in a battery of tests to characterize CNS-active drugs. (Costa et al. 1975).

MODIFICATIONS OF THE METHOD

McAllister (1992) induced spinal seizures in mice by rotating them along the body axis clockwise and anti-clockwise alternatively three times following pretreatment with a subconvulsive dose of strychnine.

Lambert et al. (1994) tested the antagonism of a glycine derivative against seizures induced by 3-mercaptopropionic acid (3-MPA).

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E.2.2.3

Picrotoxin-Induced Convulsions

PURPOSE AND RATIONALE

Picrotoxin induced convulsions are used to further evaluate CNS-active compounds. Picrotoxin is regarded as a GABA_A-antagonist modifying the function of the chloride ion channel of the GABA_A receptor complex.

PROCEDURE

Groups of 10 mice of either sex with a weight between 18 and 22 g are treated either orally or i.p. with the test compound or the standard (e. g. 10 mg/kg diazepam i.p.). Thirty min after i.p. treatment or 60 min after oral administration the animals are injected with 3.5 mg/kg s.c. picrotoxin and are observed for the following symptoms during the next 30 min: clonic seizures, tonic seizures, death. Times of onset of seizures and time to death are recorded.

EVALUATION

For time-response curves the animals receive the drug 30, 60 or 120 min prior to picrotoxin. Protection is expressed as percent inhibition relative to vehicle control. The time period with the greatest percent inhibition is said to be the peak time of drug activity. *ED*₅₀-values are calculated taking the percentage of seizures in the control group as 100%.

CRITICAL ASSESSMENT OF THE METHOD

The method has been proven to be of value amongst a battery of tests for CNS-activity.

MODIFICATIONS OF THE METHOD

Buckett (1981) describes an intravenous bicuculline test in mice (see Sect. E.3.2.6). The compound bicuculline antagonizes the action of GABA by competition on postsynaptic receptors. In the whole animal bicuculline reproducibly induces myoclonic seizures. An intravenous dose of 0.55 mg/kg was found to induce myoclonic seizures in 90–100% of mice with less than 10% mortality. GABAergic compounds such as

benzodiazepines at relatively low doses antagonize the bicuculline-induced myoclonic seizures.

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E.2.2.4

Isoniazid-Induced Convulsions

PURPOSE AND RATIONALE

Isoniazid can precipitate convulsions in patients with seizure disorders. The compound is regarded as a GABA-synthesis inhibitor (Costa et al. 1975). Clonic-tonic seizures are elicited in mice which are antagonized by anxiolytic drugs.

PROCEDURE

Ten mice of either sex with a weight of 18 to 22 g are treated with the test compound or the standard (e. g. diazepam 10 mg/kg i.p.) by oral or intraperitoneal administration. Controls receive the vehicle only. 30 min after i.p. or 60 min after p.o. treatment the animals are injected with a subcutaneous dose of 300 mg/kg isoniazid (isonicotinic acid hydrazide). During the next 120 min the occurrence of clonic seizures, tonic seizures and death is recorded.

EVALUATION

The percentage of seizures or death occurring in the control group is taken as 100%. The suppression of these effects in the treated groups is calculated as percentage of controls. *ED*₅₀-values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method has been proven to be of value amongst a battery of tests for CNS-activity.

REFERENCES AND FURTHER READING

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E.2.2.5**Yohimbine-Induced Convulsions****PURPOSE AND RATIONALE**

Antagonism against yohimbine-induced seizures in mice is considered to be a model predictive of potential anxiolytic and GABA-mimetic agents (Dunn and Fielding 1987).

PROCEDURE

Male Swiss-Webster mice (20–30 g) are individually placed in clear plastic cylinders and test compounds are administered i.p. 30 min prior to 45 mg/kg s.c. of yohimbine HCl. The animals are observed for the onset and number of clonic seizures for 60 min.

EVALUATION

ED_{50} values with 95% confidence limits are calculated for the antagonism of yohimbine-induced clonic seizures by means of the Lichtfield-Wilcoxon procedure.

CRITICAL ASSESSMENT OF THE METHOD

The antagonism against yohimbine-induced seizures can be regarded as a useful test amongst a battery of tests for anxiolytic activity.

REFERENCES AND FURTHER READING

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E.2.3**Anti-Aggressive Activity****E.2.3.1****Foot-Shock-Induced Aggression****PURPOSE AND RATIONALE**

Since the discovery of the taming effects of benzodiazepines in vicious monkeys (Heise and Boff 1961; Randall et al. 1961) tests for agents with anti-aggressivity activity have been developed for various animal species. These tests include foot-shock induced aggression in mice and rats, fighting behavior of isolated mice and aggressiveness of rats which become

extremely vicious after lesions in the septal area of the brain (Brady and Nauta 1953).

Foot-shock induced aggression is used for further characterization of centrally active drugs. Irwin et al. (1971) have attempted to compare drug classes with this method.

PROCEDURE

Male mice (NMRI, Ivanovas) with a weight between 20 and 30 g are used. Two mice are placed in a box with a grid floor consisting of steel rods with a distance of 6 mm. A constant current of 0.6 or 0.8 mA is supplied to the grid floor by a LVE constant current shocker with an associated scrambler. A 60-Hz current is delivered for 5 s followed by 5 s. intermission for 3 min. Each pair of mice is dosed and tested without previous exposure. The total number of fights are recorded for each pair during the 3-min period. The fighting behavior consists of vocalization, leaping, running, rearing and facing each other with some attempt to attack by hitting, biting or boxing. The test compound or the standard are applied either 30 min before the test i.p. or 60 min before the test orally. For a time response, the drug is given 30, 60 and 120 min prior to testing. Six pairs of drug-treated and two pairs of vehicle-treated animals are utilized for each time period. A dose range is tested at the peak of drug activity. A minimum of 3 doses (10 pairs of mice/dose) is administered for a range of doses. Control animals receive the vehicle.

EVALUATION

The percent inhibition of aggression is calculated from the vehicle control. ED_{50} -values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

Not only anxiolytics but also other classes of drugs where found to be active in this test such as sedatives like meprobamate and phenobarbital, neuroleptics such as perphenazine, analgesics such as methadone, and ethyl alcohol.

MODIFICATIONS OF THE METHOD

A survey of aggressive behavior in the rat has been given by Blanchard and Blanchard (1977).

Induction of aggressive behavior by electrical stimulation in the hypothalamus of male rats was described by Kruk et al. (1979).

Mos and Olivier (1991) reviewed the concepts in animal models for pathological aggressive behavior in human.

Play fighting, a normal behavior in several species especially in young male rats (Pellis and McKenna 1995; Pellis et al. 1997; Pellis and Pellis 1998; Pellis 2002), has been used for evaluation of drugs by Siviý et al. (1995) and Hotchkiss et al. (2003). Schneider and Koch (2005) studied the effects of chronic pubertal treatment with cannabinoids on play behavior (pins, attacks, and defense) in rats.

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E.2.3.2

Isolation-Induced Aggression

PURPOSE AND RATIONALE

Male mice, submitted to prolonged isolation, develop aggressive behavior against animals of the same sex. Compounds can be tested for their ability to suppress this isolation-induced aggression.

PROCEDURE

Male mice of NMRI-strain with an initial weight of 12 g are kept isolated in small Makrolon-cages for a period of 6 weeks. Prior to the administration of the test drug, the aggressive behavior of the animals is tested. A male mouse being accustomed to live together with other animals is placed into the cage of an isolated mouse for 5 min. Immediately, the isolated mouse will start to attack the “intruder”. The aggressive behavior of the isolated mouse is characterized by hitting the tail on the bottom of the cage, screaming and biting. The reaction time until the first attack is less than 10 s for most of the isolated mice and relatively constant for one individual. After these initial tests, the isolated mice receive the test compound, the standard drug or the vehicle either orally or subcutaneously. The aggressive behavior is evaluated 60, 120 and 240 min after oral and 30, 60 and 120 min after sc. treatment. After treatment with certain centrally acting compounds the aggressive behavior of the isolated mice is changed. The reaction time until the first attack can be prolonged or shortened. The fighting reaction can be attenuated. Then, additional mechanical stimuli can be used to elicit the fighting behavior. With highly effective drugs the aggressive behavior is completely suppressed.

EVALUATION

The number of animals with complete suppression of the fighting behavior is calculated. In animals with

diminished aggressiveness the reaction time is registered. A gradual scale of inhibition of aggressiveness is established.

CRITICAL ASSESSMENT OF THE METHOD

The fighting behavior of isolated mice is not only altered by sedative and anxiolytic compounds but also by neuroleptics and antidepressants. For example, active doses are:

| | |
|----------------|-----------------|
| Lorazepam | 2.5 mg/kg p.o. |
| Clonazepam | 2.5 mg/kg sc. |
| Haloperidol | 1.0 mg/kg p.o. |
| Chlorpromazine | 10.0 mg/kg s.c. |
| Imipramine | 25.0 mg/kg s.c. |

In this way, anti-aggressive activity of several classes can be detected.

MODIFICATIONS OF THE METHOD

Krsiak (1974, 1979) described the effects of various drugs on behavior of aggressive mice.

Olivier and van Dalen (1982) discussed the social behavior in rats and mice as an ethologically based model for differentiating psychoactive drugs.

McMillen et al. (1987) tested the effects of drugs on aggressive behavior and brain monoaminergic neurotransmission.

Krsiak (1975) showed that about 45% of single-housed male mice showed timidity instead of aggression on interactions with group-housed male mice. Several drugs, such as benzodiazepines, chlorpromazine and barbitone inhibited the isolation induced timidity without reducing other motor activities in the timid mice.

Andrade et al. (1988) tested the effect of insulin-induced hypoglycemia on the aggressive behavior of isolated mice against intruders made anosmic by application of 25 μ l of 4% zinc sulfate solution to the nasal tract 3 and 1 days before the encounters.

White et al. (1991) tested the effects of serotonergic agents and other psychotropic compounds on isolation-induced aggression in mice at doses below those which produced debilitation in the rotarod performance. Guidotti et al. (2001) used the socially isolated mouse as a model to study the putative role of allopregnenolone and 5 α -dihydroxyprogesterone in psychiatric disorders.

Francès (1988), Francès et al. (1990), Francès and Monier (1991) described an other phenomenon of isolation in rodents which can be used for evaluation of psychotropic drugs: the **isolation-induced behavioral**

deficit. Male Swiss NMRI mice were either housed in groups of 6 in home cages or isolated at the age of 4–5 weeks for 7 days. Mice were tested in pairs (one isolated and one grouped mouse) under a transparent beaker. The number of escapes was counted for the first 2 min of observation. An attempt to escape was defined as one of the following: (1) the two forepaws were placed against the wall of the beaker, (2) the mouse was sniffing, its nose at the spout of the beaker, (3) the mouse was scratching at the glass floor. Behavioral observations were taped by an observer, blind to the treatment.

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by a blind observer. Furthermore, a total of 49 different behavioral elements are scored and grouped into 7 behavioral categories: offensive, exploration, social interest, inactivity, avoidance, body care, defense.

EVALUATION

Paired and unpaired *t*-tests are used for comparisons of means of absolute values.

REFERENCES AND FURTHER READING

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E.2.3.3

Resident-Intruder Aggression Test

PURPOSE AND RATIONALE

Sijbesma et al. (1991), Mos et al. (1992), Muehlenkamp et al. (1995) studied the effects of drugs in a test for offensive aggression, the isolation-induced resident intruder aggression model (Flannelly and Lore 1975; Brain et al. 1979), in the rat.

PROCEDURE

Sprague-Dawley rats weighing 250 to 450 g are housed in a light-dark (12L:12D)-, temperature (ca. 22°C)- and humidity (ca. 55%)-controlled room.

Resident male rats (about 450 g) are tested in their home cages for aggression against a smaller (250 g) male intruder. They are treated by intraperitoneal injection of test drug or saline 15 min before the test. The resident female is removed from the cage 30 min prior to the start of the test period. After placing the intruder rat in the territorial cage, the behavior of the resident male is observed. The time until the first attack (in seconds), number of attacks, and duration of each attack (in seconds) are recorded for the next 15 min

E.2.3.4

Water Competition Test

PURPOSE AND RATIONALE

The competitive water consumption test in rats (Baenninger 1970; Syme 1974) can be used to study the influence of drugs on defensive aggression and dominance (Muehlenkamp et al. 1995).

PROCEDURE

Male Sprague-Dawley rats weighing 250 to 400 g are kept in a light-dark (12L:12D)-, temperature (ca. 22°C)- and humidity (ca. 55%)-controlled room.

Animals of equal weight are paired and housed in one cage. After 6 days, the animals are deprived of water for 23 h. Then, one water bottle is introduced with a shielded spout so that only one animal of a pair can drink at a time. Time (in seconds) and frequency of spout possession and water consumption are recorded in numbers with a special computer program for 5 min. Animals are then allowed another 55 min for water consumption. This test is repeated on 3 subsequent days. Saline or test substances are injected intraperitoneally 15 min before tests 3 and 4. The animal with

the longest duration of water consumption and frequency of spout possession is considered to be the more aggressive and/or dominant animal.

EVALUATION

Paired and unpaired *t*-tests are used for comparisons of means of absolute values, as well as means of differences of the absolute value of the two rats.

REFERENCES AND FURTHER READING

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E.2.3.5

Maternal Aggression in Rats

PURPOSE AND RATIONALE

The model of 'maternal aggression' in rats was described by Olivier et al. (1985), Olivier and Mos (1986, 1992), Mos et al. (1987, 1989). Introduction of an intruder (male or female) in the cage of a parturient female rat, induces high levels of aggression against such intruders. Maternal aggression is characterized by short latency attacks of high intensity, mostly directed toward the head or neck of the intruder and is particularly pronounced during the first part of the lactating period. The behavior can be suppressed by several drugs, e. g., 5-HT₁ agonists (Mos et al. 1990; Olivier and Mos 1992).

PROCEDURE

Female rats weighing 250–350 g are placed with a breeding male in their home cages. On the bottom of each cage an iron gauze is placed which enables the collection of ejaculation plugs. After an ejaculation plug is detected the male is left for an other week with the female after which she is placed in the observation cage provided with nesting material where she stays for the rest of the experiment. These cages are situated in an observation room under a reversed day-night rhythm. The day of birth is marked as day 0. Every parturient female is tested each day against a naive male intruder which has about 25 g less body weight than the female. Tests are performed during the first part of the dark period under red light conditions. One male intruder is placed in the female's home cage for 5 min. The ongoing behavior is videotaped and analyzed later. Each intruder is used only once and sacri-

ficed immediately afterwards with an i.p. overdose of pentobarbital, followed by shaving and describing the wounds on wound charts (Mos et al. 1984).

The aggressive behavior of the female is scored for:

- bite attack on the head (fierce biting on head and snout often causing severe wounds),
- bite attack on the body (mostly directed to the back),
- lateral threat (the animal kicks with a hindleg at the opponent),
- upright posture (accompanied by boxing),
- nipping (short and low intensity bite on the head of the opponent),
- pulling (the opponent is held by the teeth and drawn through the cage),
- lunge (very rapid movement toward the opponent, mostly followed by bite attacks),
- on top (the female holds down the opponent which lies on its back).

Besides the frequency and the duration of the elements, also the latency of the first attack and the number of attacks are recorded.

Drug experiments are performed on postpartum days 3, 5, 7, and 9 using a Latin-square design of dosage. Preceding days (day 1 and 2), intervening (4, 6 and 8) and following (10, 11, 12, and 13) days are used to establish an aggression base line and as wash-out days. Drugs are administered orally 60 min before testing.

EVALUATION

Analysis of variance is employed to detect overall significance, followed by Wilcoxon matched pairs comparison between dosages. Kruskal-Wallis analysis is used to test the differences in the bite target areas after drug treatment.

MODIFICATIONS OF THE METHOD

Postpartum aggression in rats did not influence threshold currents for electrical brain stimulation-induced aggression in rats (Mos et al. 1987).

Maternal aggression can be observed not only in rats but also in **mice** and **hamsters** (Mos et al. 1990).

Palanza et al. (1996) examined the effects of chlordiazepoxide on the differential response pattern in aggressive-naive and aggressive-experienced lactating female mice confronting intruders of either sex in a 10-min test.

Olivier et al. (1990) highlighted ethopharmacology as a creative approach to identification and characterization of novel psychotropics.

Olivier et al. (1995) gave a review on serotonin receptors and animal models of aggressive behavior.

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E.2.3.6

Rage Reaction in Cats

PURPOSE AND RATIONALE

An emotional aggressive behavior (rage reaction) can be elicited in unrestrained cats by high frequency electrical stimulation of the hypothalamus. Benzodi-

azepines have been reported (Malick 1970; Mursaki et al. 1976) to elevate the stimulus threshold for eliciting this rage reaction.

PROCEDURE

Emotional aggressive behavior is evoked by stimulation of the perifornical area of the lateral hypothalamus through chronically implanted stainless steel bipolar concentric electrodes by using threshold impulses of 1.0–2.6 mA, delivered at 50 Hz, to evoke a control attack response. Stimulation is discontinued immediately after the slowly rising current has reached threshold strength, and is performed once before drug administration as well as every 30 min thereafter. Drugs are injected by intraperitoneal route.

EVALUATION

Post-drug values are expressed as percentage of the pre-drug control value. Student's paired *t*-test is used for each time interval.

CRITICAL ASSESSMENT OF THE TEST

The method has been used for evaluation of new drugs. Most studies were devoted to the role of neurotransmitters regulating feline aggression (Siegel and Schubert 1995; Siegel et al. 1998) and the neural bases of aggression and rage in the cat (Siegel et al. 1997, 1999).

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E.2.4**Effects on Behavior****E.2.4.1****Anti-Anxiety Test (Light-Dark Model)****PURPOSE AND RATIONALE**

Crawley and Goodwin (1980) Crawley (1981) described a simple behavior model in mice to detect compounds with anxiolytic effects. Mice and rats tend to explore a novel environment, but to retreat from the aversive properties of a brightly-lit open field. In a two-chambered system, where the animals can freely move between a brightly-lit open field and a dark corner, they show more crossings between the two chambers and more locomotor activity after treatment with anxiolytics. The numbers of crossings between the light and dark sites are recorded.

PROCEDURE

The testing apparatus consists of a light and a dark chamber divided by a photocell-equipped zone. A polypropylene animal cage, 44 × 21 × 21 cm, is darkened with black spray over one-third of its surface. A partition containing a 13 cm long × 5 cm high opening separates the dark one third from the bright two thirds of the cage. The cage rests on an Animex activity monitor which counts total locomotor activity. An electronic system using four sets of photocells across the partition automatically counts movements through the partition and clocks the time spent in the light and dark compartments. Naive male mice or rats are placed into the cage. The animals are treated 30 min before the experiment with the test drugs or the vehicle intraperitoneally and are then observed for 10 min. Groups of 6–8 animals are used for each dose.

EVALUATION

Dose-response curves are obtained and the number of crossings through the partition between the light and the dark chamber are compared with total activity counts during the 10 min.

CRITICAL ASSESSMENT OF THE METHOD

It has been shown that a variety of anxiolytics, including diazepam, pentobarbital and meprobamate produce a dose-dependent increase in crossings, whereas non-anxiolytic agents do not have this facilitatory effect. Furthermore, the relative potency of anxiolytics in increasing exploratory behavior in the two-compartment chamber agrees well with the potency found in clinical trials.

The test has the advantage of being relatively simple with no painful stimuli to the animals. The specificity of the method remains open.

MODIFICATIONS OF THE METHOD

Using a similar method, called black and white test box, Costall et al. (1987, 1988, 1989) studied the effects of anxiolytic agents and reported an anxiolytic effect of dopamine receptor antagonists, such as sulpiride and buspirone.

Sanchez (1995) presented a fully automated version of the black and white two-compartment box for mice.

Barnes et al. (1992) used this model to study the interaction of optical isomers modifying rodent aversive behavior.

Kilfoil et al. (1989) used a similar apparatus to test compounds for anxiogenic and anxiolytic activity.

Animal models of anxiety and their relation to serotonin-interacting drugs have been reviewed by Broekkamp et al. (1989) and by Griebel (1995).

Laboratory rats prefer to dwell on a solid floor rather than an grid one, particularly when resting. Manser et al. (1996) described an operant test in rats to determine the strength of preference for flooring. The apparatus consisted of a grid-floored cage and a solid-floored cage, joined via a central box containing a barrier whose weight was adjustable. The rats had to lift the barrier in order to explore the whole apparatus or were confined on the grid floor and then had to lift the barrier in order to reach the solid floor.

Hascoët and Bourin (1998) tested anxiolytic and psychostimulant drugs in a fully automated and computer-integrated two-compartment light/dark box.

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E.2.4.2

Anticipatory Anxiety in Mice

PURPOSE AND RATIONALE

When group-housed mice are removed one by one from their home cage, the last mice removed have always higher rectal temperatures than those removed first (Borsini et al. 1989; Lecci et al. 1990). This phenomenon is interpreted as being caused by anticipatory fear for an aversive event (handling causes stress-induced hyperthermia). Consequently, this test is thought to be a model of anticipatory anxiety. The anticipatory increase in temperature was prevented by prior treatment with diazepam and buspirone, whereas several other drugs did not affect this phenomenon (Lecci et al. 1990). The usefulness of this model for obsessive-compulsive disorder in man is discussed.

PROCEDURE

Groups of 18 male albino Swiss mice weighing 25–30 g are housed at constant room temperature and relative humidity for at least 7 days in Makrolon cages to adapt to the environment. Test drugs or standard (diazepam) or solvent are administered orally in various doses to groups of 18 mice prior to the test. Thirty min later, the first 3 mice are removed from the cage and the rectal temperature registered by inserting a silicone lubricated thermistor probe (2 mm diameter) for 2.5 cm into the rectum. The average temperature of these 3 mice is taken as basal value. Mice number 4 through 15 are simply removed and again returned to the cage, and thereafter body temperature is determined in the remaining three animals. The difference of the mean value of these mice and the basal values is calculated as increase. Vehicle treated test groups display increases of 1.1 to 1.3°C.

EVALUATION

The mean increase values of treated groups \pm SEM are compared by ANOVA statistics with the controls.

MODIFICATIONS OF THE METHOD

Van der Heyden et al. (1997) adapted the group-housed stress-induced hyperthermia paradigm to single housed animals in order to drastically reduce the number of animals used. Repeated, but not single, disturbance of animals resulted in a strong hyperthermia (Δt) within 10 min. The final test paradigm chosen involved repeated temperature measurements at 10 min intervals, thus providing both information on basal temperature and Δt in each animal within a short time frame.

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E.2.4.3

Social Interaction in Rats

PURPOSE AND RATIONALE

In an unfamiliar and brightly lit environment, the normal social interaction of rats (e. g. sniffing, nipping, grooming) is suppressed. Anxiolytics counteract this suppression.

PROCEDURE

Male Sprague-Dawley rats (225–275 g body weight) are housed in groups of 5 animals. The apparatus used for the detection of changes in social behavior and exploratory behavior consists of a Perspex open-topped box (51 × 51 cm and 20 cm high) with 17 × 17 cm marked areas on the floor. One hour prior to the test, two naive rats from separate housing cages are treated with the test compound orally. They are placed into the box (with 60 W bright illumination 17 cm above) and their behavior is observed over a 10-min period by remote video recording. Two types of behavior can be noted:

- social interaction between the animals is determined by timing the sniffing of partner, crawling under or climbing over the partner, genital investigation of partner, and following partner,
- exploratory motion is measured as the number of crossings of the lines marked on the floor of the test box.

Six pairs are used for each dose.

EVALUATION

The values of treated partners are compared with the data from 6 pairs of untreated animals using single factor analysis of variance followed by Dunnett's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that there may be some analogy between “social anxiety” in humans and the behavior of rats in the social interaction test, there appear some potential complications with this test, such as an increase of social interaction after anxiolytics independent from the environment, dependence on external variables such as time of the day, and the complicated nature of social interaction.

MODIFICATIONS OF THE TEST

Sams-Dodd (1995) described the automation of the social interaction test in rats by a commercially available video-tracking system.

Gheusi et al. (1994) studied the effects of tetrahydroaminoacridine (THA) on social recognition in rats indicating a dissociation of cognitive versus non-cognitive processes.

Doses of 1.0 to 4.0 mg/kg i.p. phencyclidine (PCP) reduce the social interaction time in rats in a dose-dependent fashion. Reversal of the PCP-induced social withdrawal has been used as an animal model for neuroleptic resistant schizophrenia (Carlsson and Carlsson 1990; Corbett et al. 1995).

Olfactory investigation of ovariectomized females by adult male mice decreases during repeated confrontations with the same female intruder, whereas aggressive behavior gradually increases (Winslow and Camacho 1995). Administration of scopolamine blocked decrements in olfactory investigation in repeated confrontations and significantly reduced aggression. Acetylcholinesterase inhibitors enhanced the rate of decrement of olfactory investigation, but had differential effects on aggression.

Wongwitdecha and Marsden (1996) investigated the effects of isolation rearing on anxiety using the social interaction paradigm and compared the effects of diazepam on social interactive behaviors in isolation and socially reared rats.

Sams-Dodd (1997) studied the effect of novel anti-psychotic drugs on phencyclidine-induced stereotyped behaviour and social isolation in the rat social interaction test.

The rat social interaction model has been used by various authors to characterize the potential anxiolytic effects of serotonin receptor antagonists, such as 5-HT_{1C} receptors antagonists (Kennett et al. 1989; Kennett 1992), 5-HT₂ receptor antagonists (Kennett et al. 1994, 1995, 1996a, b, 1997; Costall and Naylor 1995), 5-HT₃ receptor antagonists (Costall et al. 1990; Costall and Naylor 1992; Blackburn et al. 1993), 5-HT₄ receptor antagonists Kennett et al. (1997), cholestyramine receptor antagonists (Hughes et al. 1990; Costall et al. 1991; Singh et al. 1991) and of nitric oxide synthase inhibitors (Volke et al. 1997).

File and Johnston (1989) reported a lack of effects of 5-HT₃ receptor antagonists in the social interaction test in the rat.

Woodall et al. (1996) described a **competition procedure** in rats. On the first week of experimentation groups of 3 rats (triads) are familiarized with the test box and sweetened milk from a drinking spout located

on the end wall. The drinking spout is surrounded by a Perspex tube (4.5 cm diameter) which ensures that only one animal is able to drink at a time. All animals are deprived of water overnight and on the following day placed into the testing box and given access to the sweetened milk for 15 min. On the second week of testing, the rats are not longer water deprived and are given access to the testing box and sweetened milk for 5 min. During the testing period, the rats are observed every 5 s, and a note is made which animal is drinking. This procedure is carried out twice a week for a period of 5 weeks. Drugs are administered to either the dominant or subordinate rat in each triad 15 min prior to testing. Following the drug study, all triads are tested for 2 trials to ensure that their rank orders return to baseline levels. The access of the subordinate member to sweetened milk is increased after administration of an anxiolytic drug.

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- investigator on alternate days to reduce stress. Groups consist of 6 rats for each dose. Thirty min after i.p. administration of the test drug or the standard, the rat is placed in the center of the maze, facing one of the enclosed arms. During a 5 min test period the following measures are taken: the number of entries into and time spent in the open and enclosed arms; the total number of arm entries. The procedure is conducted preferably in a sound attenuated room, with observations made from an adjacent room via a remote control TV camera.

EVALUATION

Motor activity and open arm exploratory time are registered. The values of treated groups are expressed as percentage of controls. Benzodiazepines and valproate decrease motor activity and increase open arm exploratory time.

CRITICAL ASSESSMENT OF THE METHOD

The method is rather time consuming, but can be regarded as a reliable measure of anxiolytic activity. Computerized automatic elevated plus maze systems may help to overcome these difficulties (TSE Systems, Bad Homburg, Germany).

MODIFICATIONS OF THE METHOD

Latency to enter a mirrored chamber by mice has been described as a behavioral assay for anxiolytic agents (Toubas et al. 1990).

Handley and McBlane (1993) provided an assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs.

Lapin (1995) studied the effect of handling, sham injection, and intraperitoneal injection of saline on the behavior of mice in an elevated plus-maze. These procedures produce behavior considered to be typical for anxiety inducing drugs. Saline-treated groups taken as controls possess the behavioral profile of stressed and anxious animals.

Pokk et al. (1996) described a method of small platform-induced stress whereby mice were individually placed for 24 h on a small platform (3 cm high, 3.5 cm in diameter) which was fixed at the center of a plastic chamber (20 cm diameter, 40 cm high) and was surrounded by water (1 cm deep) at 22°C.

Shepherd et al. (1994) described the **“elevated zero-maze”** as a modification of the elevated plus-maze model of anxiety in rats which incorporates both traditional and novel ethological measures in the analysis of drug effects. The design comprises an elevated annular platform with two opposite enclosed quadrants

E.2.4.4

Elevated Plus Maze Test

PURPOSE AND RATIONALE

Out of many possibilities to modify maze tests (e. g. water maze (Danks et al. 1991), Y-maze, radial maze (Di Cicco 1991)) the **elevated plus maze** (Montgomery 1958; Pellow et al. 1985; Corbett et al. 1991) has found acceptance in many laboratories (Liebisch et al. 1998; Landgraf et al. 1999; Schwarzberg et al. 1999; Keck et al. 2001; Brakebusch et al. 2002). The test has been proposed for selective identification of anxiolytic and anxiogenic drugs. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time; anxiogenic compounds have the opposite effect.

For effects on learning and memory see Sect. F.3.3.3.

PROCEDURE

The plus-maze consists of two open arms, 43 × 15 cm (L × W), and two enclosed arms, 43 × 1523 cm (L × W × H), open to the top, arranged so that the two open arms are opposite to each other. The maze is elevated to a height of 70 cm (TSE Systems, Bad Homburg, Germany). The rats (200–250 g body weight) are housed in pairs for 10 days prior to testing in the apparatus. During this time the rats are handled by the

and two open, removing any ambiguity in interpretation of time spent in the central square of the traditional design and allowing uninterrupted exploration. A similar equipment, built for mice (TSE Systems, Bad Homburg, Germany), was used by Cryan et al. (2004) and Korsgaard et al. (2005).

Jardim et al. (1999) evaluated the elevated T-maze as an animal model of anxiety in the mouse and found important differences between mice and rats.

Based on their plus-maze behavior, that is the time spent in the open arms, Ho et al. (2002) divided male Wistar rats into two subgroups with either “low” or “high” anxiety.

Silva and Frussa-Filho (2000) recommended the plus-maze discriminative avoidance task as a model to study memory–anxiety interactions. Mice are conditioned to choose between two enclosed arms (in one of which light and noise are presented as aversive stimuli) while avoiding the two open arms of the apparatus. The test has the advantage of measuring, at the same time and in the same animals, learning/memory (by the percentage of time spent in aversive closed arm) and anxiety (by the percentage of time spent in the open arms).

Montag-Sallaz and Montag (2003) tested cognitive and motor coordination deficits in Tenascin-R-deficient mice on an elevated plus maze. For 5 min, the behavior was recorded on videotape and the numbers of entries into the central part, the closed or the open arms were counted and the time spent in these departments was determined using the VideoMot 2 system (TSE Systems, Bad Homburg, Germany).

The same system was used by Karl et al. (2003) to study behavioral effects of neuropeptide Y in F344 rat substrains with reduced dipeptidyl-peptidase IV activity.

Korte and De Boer (2003) recommended fear-potentiated behavior in the elevated plus-maze in rats as a robust animal model of state anxiety.

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E.2.4.5

Water Maze Test

PURPOSE AND RATIONALE

Spatial learning of rats can be tested in a water maze as described by Morris (1984) and McNaughton and Morris (1987).

For effects on learning and memory see Sect. F.3.3.5.

PROCEDURE

The water maze consists of a circular tank with 100 cm diameter and a wall 20 cm above the water level. A circular platform (9 cm diameter, covered with white linen material for grip) is hidden 2 cm below the water level. The water is made opaque using titanium dioxide

suspension and is kept at about 23°C during the experiment. Training takes place on three consecutive days, with the rats receiving 4 consecutive trials per day with an inter-trial interval of 6–10 min. Each trial is started from one of four assigned polar positions with a different sequence each day. The latency to find the platform is measured as the time of placement of the rat in the water to the time it finds the platform. If the animal fails to find the platform in any trial within 3 min it is placed on it for 10 s.

EVALUATION

On day four a probe test is performed. The platform is removed and the time spent in the target quadrant (the quadrant in the center of which the platform has been located) and the number of annulus crossings (across the actual location where the platform has been located) in the first 60 s of exposure are measured. The time to the first annulus crossing is also taken as a measure of performance on the 13th (i. e. probe) trial.

Buspirone (Rowan et al. 1990) as well as benzodiazepines (McNaughton and Morris 1987) increase the latency to find the platform in the training period and impair the number and the time of annulus crossings.

CRITICAL ASSESSMENT OF THE METHOD

The water maze test measures learning and memory rather than the anxiolytic activity. If the test is used for memory, rats which have learned quickly and consistently to find the platform are kept in their cages for 2 weeks and then re-tested. Rats which solve the escape immediately, are considered to have retained memory (see also Sect. F.3.3.5).

MODIFICATIONS OF THE METHOD

Bane et al. (1996) used the Morris water maze to study the adverse effects of the non-competitive NMDA receptor antagonist dextromorphan on the spatial learning of rats.

Van der Staay (2000) studied the effects of the size of the Morris water tank on spatial discrimination learning in the CFW1 mouse. Mice can best be tested in a small pool, because the time and distance swam to find and escape onto the platform are decreased and the probability of success is increased.

Schmitt and Hiemke (2002) reported that a γ -aminobutyric acid transport inhibitor impairs spatial learning in the Morris water maze.

Winter et al. (2004) determined long-term functional outcome after mild focal cerebral ischemia by tracking swimming performance in a Morris water

maze with the computer-based VideoMot2 system (TSE Systems, Bad Homburg, Germany).

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E.2.4.6

Staircase Test

PURPOSE AND RATIONALE

The staircase test for evaluating anxiolytic activity was originally described for rats by Thiebot et al. (1973). When introduced into a novel environment, rodents experience a conflict between anxiety and exploratory behavior manifested by increased vigilance and behavioral activity. In the staircase paradigm, step-climbing is purported to reflect exploratory or locomotor activity, while rearing behavior is an index of anxiety state. The number of rearings and steps climbed are recorded in a 5 min period. The dissociation of these parameters is considered to be characteristic for anxiolytic drugs. The test was modified for rapid screening of anxiolytic activity in mice (Simiand et al. 1984).

PROCEDURE

For experiments with mice the staircase is composed of five identical steps 2.5 cm high, 10 cm wide and 7.5 cm deep. The internal height of the walls is constant along the whole length of the staircase. Naive

male mice (Charles River strain) with a weight between 18 and 24 g are used. Each animal is used only once. The drug or the standard is administered orally 1 h or 30 min subcutaneously before the test. The animal is placed on the floor of the box with its back to the staircase. The number of steps climbed and the number of rearings are counted over a 3-min period. A step is considered to be climbed only if the mouse has placed all four paws on the step. In order to simplify the observation, the number of steps descended is not taken into account. After each test, the box has to be cleaned in order to eliminate any olfactory cues which might modify the behavior of the next animal.

EVALUATION

Twelve mice are used for the untreated control group, each drug group, and for the group receiving the standard. The average number of steps and rearings of the control group is taken as 100%. The values of treated animals are expressed as percentage of the controls.

CRITICAL ASSESSMENT OF THE METHOD

The staircase test has been proven as a simple and reliable method for screening of anxiolytics in several laboratories. Many applications and modifications have been described in the literature (Hourri 1985; Steru et al. 1987; Keane et al. 1988; Emmannouil and Quock 1990; Simiand et al. 1993).

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E.2.4.7**Cork Gnawing Test in the Rat****PURPOSE AND RATIONALE**

Cork gnawing behavior in the rat has been proposed as a screening method for buspirone-like anxiolytics by Pollard and Howard (1991).

PROCEDURE

Adult male Evans rats serve as subjects. They are housed 4 per cage on a regular light/dark cycle with free access to food and water except for the period between injection and the end of a test session. For the test session one animal is placed in a stainless steel cage with wire mesh bottom. A session consists of placing the subject in the test cage with a cork stopper weighing between 2–3 g for 30 min. Initially, the amount gnawed is relatively high and variable within and between subjects. After 30 training sessions, the amount is low and stabilized. The test compounds are injected 30 min before the test and food is withdrawn.

EVALUATION

Each cork is weighed to the nearest 0.01 g before and after the session. The average cork loss during the previous control days is taken as baseline and the amount after drug treatment is expressed as percentage of baseline. Buspirone-related compounds as well as benzodiazepines and meprobamate show a dose dependent increase of cork gnawing, but amphetamine, chlorpromazine, imipramine and morphine do not.

CRITICAL ASSESSMENT OF THE METHOD

The test is worthwhile to be mentioned, since buspirone – whose anxiolytic action was discovered during clinical trials to assess possible antipsychotic action and not by use of animal tests for anxiolysis – is active in this test but not in most other classical tests for anxiolytic activity.

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screening method for anxiolytic drugs by Gardner (1985).

PROCEDURE

Wistar rat pups are bred on site and left undisturbed with their mother, except for cage-bedding replacement, until the day of testing. The pups are tested at 9–12 days of age. On the day of testing the pups are separated from their mother and taken in their home cage to the quiet experimental room. In the morning all pups are subjected to handling stress and the magnitude of their ultrasound emission is observed. The stress consists of holding the pup by the base of the tail, between forefinger and thumb of the experimenter, and thus suspending it 5 cm above the bench for 30 s. A prior control recording (30 s) is taken when the pup is held gently in the experimenter's hand, whereby the pups emit only a few ultrasounds. Responses when held by the tail are more than 10 times higher. This entire hand-holding-tail-holding procedure is immediately repeated. Ultrasounds are recorded with suitable detectors with 42 kHz as the center of a 10 kHz recording range. The output of the detectors is fed into pen recorders. The total number of ultrasonic cries in the two sessions of hand holding and the two sessions of tail holding are calculated and used as the control activity of each pup. Any pup producing a total of less than 50 ultrasounds when held by the tail is excluded from the drug study. The pups are kept in the home cage in the test laboratory until the afternoon. Three to four hours after the first test the pups are randomly allocated to several equally sized groups, weighed, marked, and dosed intraperitoneally either with the vehicle or drug and placed back in the home cage. Thirty min after dosing, each pup is subjected to the same handling stress as that used in the morning session, and the total number of sounds produced is calculated in the same way.

EVALUATION

The afternoon response to tail holding is expressed as a factor of the morning response. The mean factor for the saline-treated animals is taken to be 100% in calculations of percentage changes in ultrasound emission by drugs.

E.2.4.8**Distress Vocalization in Rat Pups****PURPOSE AND RATIONALE**

Measurement of ultrasonic vocalization induced by tail-holding in rat pups was proposed as a simple

CRITICAL ASSESSMENT OF THE METHOD

Anxiolytic benzodiazepines dose-dependent inhibit vocalization. Amitriptyline and haloperidol have no effect. Chlorpromazine, muscimol and prazosin reduce sound at doses which also induce overt sedation.

Therefore, the method can be regarded as relatively specific for anxiolytic activity.

MODIFICATIONS OF THE METHOD

Kehne et al. (2000) used the ULTRAVOX system (Noldus Information Technology, Wageningen, the Netherlands) to detect and quantify separation-induced ultrasonic vocalization in rat pups.

Using this equipment, Siemiatkowski et al. (2001) reported opposite effects of olanzapine and haloperidol in the rat ultrasonic vocalization test.

Molewijk et al. (1996), Griebel et al. (2002), and Steinberg et al. (2002) used maternal separation-induced distress vocalization in **guinea pig** pups to test anxiolytic and antidepressant drugs.

Rupniak et al. (2000) investigated the stress-induced vocalizations by central NK₁ receptors using pharmacological antagonists in guinea pigs, a species with human-like NK₁ receptors, and transgenic NK1R^{-/-} mice.

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E.2.4.9

Schedule Induced Polydipsia in Rats

PURPOSE AND RATIONALE

Food deprived rats exposed to a procedure in which food is delivered intermittently will drink large amounts of water if given the opportunity to do so. This behavioral phenomenon is termed schedule-induced polydipsia and is an example of a more general class of behaviors termed adjunctive behaviors (Falk 1971; Pellon and Blackman 1992). Adjunctive behaviors have been cited as potential animal models of human obsessive-compulsive disorders (Pitman 1989).

PROCEDURE

Male Wistar rats weighing 180–250 g are individually housed at a 12 h/12 h light/dark cycle for a 1 week acclimation period with free access to food and water. Then they are placed on a restricted diet which maintains 80% of their free feeding body weight. To induce polydipsia, rats are placed in test chambers housed in sound attenuated boxes where a pellet dispenser automatically dispenses two 45 mg pellets on a fixed time 60-s (FT-60s) feeding schedule over a 150 min test session. Water is available at all times in the test chambers. After 4 weeks exposure to the FT-60s feeding schedule, approximately 80% of the rats meet the predetermined criterion for water consumption (greater than 60 ml water per session) and are considered to have polydipsic behavior.

Rats receive the test compounds in various doses daily or the vehicle intraperitoneally 60 min prior to testing. They are tested once a week to assess schedule induced polydipsia. Water bottles are weighed before and after the 150-min test sessions.

EVALUATION

The experimental data comparing the effects of chronic administration of compounds on schedule-induced polydipsia are analyzed with the Mann Whitney *U*-test.

MODIFICATIONS OF THE METHOD

Yadin et al. (1991) proposed spontaneous alternation behavior in rats as an animal model for obsessive-compulsive disorder. Food-deprived rats were run on a T-maze in which both a white and a black goal box were equally baited with a small amount of chocolate milk. Each rat was given 7 trials every other day during which it was placed in the start box and allowed to make a choice. The mean number of choices until an alternation occurred was recorded. After a baseline of spontaneous alternation was achieved the rats were treated with the non-selective serotonin agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) (1.25 mg/kg i.p.) or the selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT) (2 mg/kg i.p.) which both disrupted the spontaneous alternation. A course of chronic treatment (2 × 5 mg/kg for 21 days) with the selective 5-HT uptake blocking agent fluoxetine had a protective effect on the 5-MeODMT-induced disruption of spontaneous alternation behavior. The authors speculated that serotonergic manipulations of spontaneous alternation may be a simple animal model for the perseverative symptoms or indecisiveness seen in patients with obsessive-compulsive disorder.

Bös et al. (1997) and Martin et al. (1998) tested agonists of 5HT_{2C} receptors in the schedule-induced polydipsia task in rats and proposed them as improved therapeutics for obsessive compulsive disorder.

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E.2.4.10**Four Plate Test in Mice****PURPOSE AND RATIONALE**

The four plate test in mice has been described by Aron et al. (1971), Boissier et al. (1968) as a method for the rapid screening of minor tranquilizers.

PROCEDURE

The test box has the shape of a rectangle (25 × 18 × 16 cm). The floor is covered with 4 identical rectangular metal plates (8 × 11 cm) separated from one another by a gap of 4 mm. The plates are connected to a source of continuous current which applies to 2 adjacent plates a mild electrical shock of 0.35 mA for 0.5 s. This evokes a clear flight reaction of the animals.

Adult male Swiss albino mice, weighing 17 to 23 g, are randomly divided into different groups. Thirty min before the test the animals are injected intraperitoneally with the test drug or the vehicle.

At the beginning of the test, the mouse is gently dropped onto a plate and is allowed to explore the enclosure for 15 s. After this, every time the animal crosses from one plate to another, the experimenter electrifies the whole floor for 0.5 s, which evokes a clear flight-reaction of the mouse which often crosses 2 or 3 plates. If it continues running, no new shock is delivered during the following 3 min.

EVALUATION

The number of times the apparatus is electrified is counted each minute for 10 min. The delivery of shocks decreases dramatically the motor activity. The number of shocks received during the first min is taken as parameter. This number is increased by minor tranquilizers, such as benzodiazepines, but not by neuroleptics and psychoanaleptics.

CRITICAL ASSESSMENT OF THE METHOD

The test is of value to differentiate minor tranquilizers, such as benzodiazepine anxiolytics, from neuroleptics. However, some stimulants (e. g. amphetamine) produce an increase in punished plate crossings and some anxiolytics do not.

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E.2.4.11**Foot-shock-Induced Freezing Behavior in Rats****PURPOSE AND RATIONALE**

Footshock-induced freezing behavior in rats has been proposed as a model for anxiolytics by Conti et al. (1990).

PROCEDURE

Male Sprague-Dawley rats with a weight between 200 and 350 g are used. The animals receive a single i.p. injection of the test compound or the vehicle 30 min prior to being placed in a standard conditioning chamber (e. g., Coulbourn Instruments) for a 6.5 min session. Two and 2.5 min after the start of the session, a scrambled footshock (0.5 mA, 0.5 s) is delivered through the grid floor of the chamber. Using an assembly of push buttons interfaced with a computer, an observer monitors the amount of time each animal spends engaged in the following mutually exclusive behaviors:

- Freezing: immobility with rigid body posture
- Sedated posture: sitting or sleeping
- Small exploratory movements: movements involving the torso or front paws only, vertical movements of the head, or sniffing.

- Locomotion: activity involving hind paws, grooming or rearing.

Frequency of rearing is also counted. All behaviors are monitored for the entire 6.5 min session.

EVALUATION

Duration of foot-shock induced freezing after the second shock is taken as the critical parameter. Time spent in freezing posture after administration of test compounds is compared with the controls. Anxiolytics like diazepam and buspirone show dose-dependent effects, but not haloperidol.

CRITICAL ASSESSMENT OF THE METHOD

The method seems to discriminate anxiolytics including buspirone from other centrally acting drugs.

MODIFICATIONS OF THE METHOD

Footshock-induced ultrasonic vocalization has been suggested as another model of anxiety (Tonoue et al. 1986; Kaltwasser 1990; Miczek et al. 1991; De Vry et al. 1993; Nielsen and Sánchez 1995; Schreiber et al. 1998). Test cages (22 × 22 × 22 cm) made of grey Perspex are equipped with a metal grid floor (distance between the bars = 1 cm). Electric footshocks are delivered from a 12 bit programmable shock source and ultrasounds are picked up by a microphone (range 18–26 Hz) placed in the center of the cage lid. The total vocalization time is measured. The rats are placed individually in the test cages and immediately receive a series of 0.5 mA inescapable footshocks each of 1 s duration with a shock interval of 5 s. The vocalization is measured for a 10 min period starting 1 min after the last shock. Drugs or saline are given subcutaneously 30 min before the test.

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E.2.4.12

Experimental Anxiety in Mice

PURPOSE AND RATIONALE

Ogawa et al. (1966, 1990, 1993) designed a communication box to induce experimental anxiety in mice by employing intraspecies emotional communication. The inside of the communication box was divided into foot-shock and non-foot-shock compartments by transparent plastic boards. The animals, which were individually placed into each compartment, were unable to make physical contact with one another, but were able to receive other cues such as visual, auditory and olfactory sensations. During the foot shock period, the animals placed in the non-foot-shock compartments were exposed to the emotional cues from foot-shocked animals, such as shrieks, smell of feces or urine, and jumping response.

PROCEDURE

The floor of the communication box is equipped with grids for electric shock. The inside is divided into small compartments (10×10 cm), consisting of foot-shock compartments with a grid floor and non-foot-shock compartments with a grid floor covered by transparent plastic boards. The foot-shock compartments are arranged such as to surround the non-foot shock compartments.

The experimental groups consist of the following 3 groups: sender group, responder group, and food-yoked group to responder. Sender animals receive a foot shock of 10-s duration at intervals of 50 s for 3 h. The electric current for the shock is increased stepwise from 1.6 mA to 2.0 mA at a rate of 0.2 mA per 1 h. Responders are exposed daily to the emotional responses of sender animals, 3 h per day for 3 days. Sender animals are changed daily to naive mice to prevent a reduced emotional response to foot shock based on adaptation or learned helplessness due to repeated exposure. Both sender and responder animals are placed individually in each compartment of the communication box 15 min before beginning of the shock period. On day-1, responder animals are returned to their home cages after the 3-h foot shock period. On day-2, after completing the foot-shock period, they are transferred to metal cages and are housed

in the cages with 4 animals per cage under food-deprivation condition. Food-yoked control animals are maintained to the metal cage during the foot-shock period under the aggregated housing condition (5 animals each) and then they are returned to the home cages after the foot shock period. From beginning of the day-2 experiment to completion of the day-3 experiment, they are maintained in the metal cages under aggregating housing. On day-3, just after completing the foot-shock period, the responders are sacrificed by chloroform, and their stomachs are removed. The stomachs are visually inspected for lesions.

Drugs are administered orally at different doses either with a single dose on day-3 or daily 30 min before the shock period.

EVALUATION

Data are reported as the incidence of mice with gastric lesions characterized by slight erosions or bleeding. Active anxiolytics reduce the incidence of gastric ulcers found in food-deprived animals. The incidence in food-yoked animals is much lower. The data are analyzed by Fisher's exact probability test.

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E.2.4.13

mCPP-Induced Anxiety in Rats

PURPOSE AND RATIONALE

The metabolite of the antidepressant drug trazodone 1-(3-chlorophenyl)piperazine (=mCPP), classified as 5-HT_{1C} agonist (Rocha et al. 1993; Gibson et al. 1996) or 5-HT_{1B/2C} agonist (Dryden et al. 1996), has been shown to be anxiogenic both in man and in rats (Curzon et al. 1991). The compound induces hypophagia (Samanin et al. 1979; Dryden et al. 1996; Yamada et al. 1996) and hypolocomotion (Kennett et al. 1996, 1997a), inhibits social interaction in rats, diminishes exploratory activity of rats in the open field test (Czyrak et al. 1994; Meert et al. 1997) and in the light-dark box test (Bilkei-Gorzo et al. 1998), induces hyperthermia (Aulakh et al. 1995; Kennett et al. 1997b)

and reduces ultrasound-induced defensive behavior in the rat (Beckett et al. (1996). Antagonism against these symptoms has been proposed as a screening model for anxiolytic drugs (Bilkei-Gorzo et al. 1996, 1998; Wallis and Lal 1998).

PROCEDURE

Male Sprague Dawley rats (220–250 g) are housed in groups of 6 under a 12 h light/dark cycle with free access to food and water.

mCPP-Induced Locomotion

Rats are placed in a room adjacent to the experimental room on the day of the procedure. They are dosed either orally 1 h, or i.p. 30 min before the locomotion test with test compound or vehicle, and injected 20 min before the test with 7 mg/kg mCPP i.p. or saline in groups of four. Rats are returned to their home cages after dosing. At 0 h they are each placed in automated locomotor activity cages made of black Perspex with a clear Perspex lid and sawdust covered floor under red light for 10 min. During this time, locomotion is recorded by means of alternately breaking two photocell beams traversing opposite ends of the box 3.9 cm above floor level.

mCPP-Induced Hypophagia

Rats are individually housed on day 1 and on day 3 they are deprived of food. Twenty-three hours later, they are orally treated with the test drug or vehicle and returned to their home cages. Forty min later, they are given 5 mg/kg mCPP or saline i.p. and again returned to their home cages. After a further 20 min, weighted amounts of their normal food pellets are placed in their food hoppers and the amount remaining after 1 h is measured.

EVALUATION

The effect of the test compound on mCPP-induced hypolocomotion is determined by one-way ANOVA and Newman-Keuls test. The dose producing 50% disinhibition of mCPP is also estimated. Feeding test data are subjected to one-way ANOVA and Dunnett's test.

MODIFICATIONS OF THE METHOD

Griebel et al. (1991) described neophobic and anxious behavior in mice induced by m-CPP.

Czyrak et al. (1994) measured the antagonism of anti-psychotics against the mCPP-induced hypothermia in mice.

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E.2.4.14

Acoustic Startle Response in Rats

PURPOSE AND RATIONALE

The acoustic startle reflex is a relatively simple behavior that occurs naturally in mammals and is affected by a variety of treatments. It consists of a series of rapid movements beginning at the head and moving caudally involving contraction and extension of major muscle groups in response to auditory stimuli with a rapid onset, or rise time. Responses are graded in amplitude in relation to stimulus intensity, and may show habituation and sensitization. Startle response can be used to determine sites and mechanisms of drug action (Davis 1982).

PROCEDURE

Male Wistar rats weighing about 200 g are used. Acoustic startle reflexes are measured in a specially build apparatus, e. g., Coulbourn Instruments Acoustic Response Test System or TSE Systems, Bad Homburg, Germany. The animals are individually placed in 8 × 8 × 16 cm open air cages that restrict locomotion but do not immobilize the animal, and are placed on one of four platforms within a sound-attenuating acoustic chamber. A ventilating fan provides an ambient noise level. Acoustic stimuli consist of white noise bursts lasting 20 ms at 98 dB and 124 dB SPL. Simultaneously with the rapid onset of each stimulus, the animal's physical movement within the cage on the platform is measured for 200 ms as an electrical voltage change via a strain gauge which is converted to grams of weight change following analog to digital conversion. Data are recorded automatically by an interfaced microcomputer.

Pre-tests are performed with all animals to obtain control values. The animals are treated 2 h prior the experiment with test drugs or vehicle given orally or subcutaneously.

EVALUATION

The results are given as percentage of the change, related to the values obtained in the pre-test and as-

sessed by a one-way ANOVA, followed by Dunnett's test when appropriate.

MODIFICATIONS OF THE METHOD

The test has been modified in various ways, e. g., inhibition by a prepulse (Keith et al. 1991; Rigdon and Viik 1991; Taylor et al. 1995) or fear-induced potentiation (Davis 1986, 1992).

Schulz et al. (1996) performed acoustic startle experiments in rats with a potent and selective non-peptide antagonist of the corticotropin releasing factor receptors.

Walker and Davis (1997) found that the amplitude of acoustic startle response in rats was increased by high illumination levels.

Devices to register the intensity of fear-potentiated startle response in rats were described by Hijzen et al. (1995). Yilmazer-Hanke et al. (2002) studied fear-potentiated startle and exploration-related anxiety in inbred Roman high- and low-avoidance rats.

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E.2.4.15

Unconditioned Conflict Procedure (Vogel Test)

PURPOSE AND RATIONALE

Vogel et al. (1971) described a simple and reliable conflict procedure for testing anti-anxiety agents. Thirsty, naive rats were administered shocks while licking water.

PROCEDURE

The apparatus is a clear Plexiglas box (38 × 38 cm) with a black Plexiglas compartment (10 × 10.5 cm) attached to one wall and an opening from the large box to the small compartment. The entire apparatus has a stainless-steel grid floor. A water bottle with a metal drinking tube is fitted to the outside of the small compartment, so that the tube extended into

the box at a height 3 cm above the grid. Rats lick in bursts with a relatively constant rate of 7 licks per sec. A drinkometer circuit is connected between the drinking tube and the grid floor of the apparatus, so that the rat completes the circuit whenever it licks the tube. Shock is administered to the feet of the animal by switching the connections to the drinking tube and grids from the drinkometer to a shocker which applies an unscrambled shock between the drinking tube and the grid floor.

Naive adult male rats are used. Thirty min after intraperitoneal injection, the rat is placed in the apparatus and allowed to find the drinking tube and to complete 20 licks before shock (available at the tube for 2 s) is administered. The rat controls shock duration by withdrawing from the tube. A 3-min timer is automatically started after the termination of the first shock. During the 3-min period, shocks are delivered following each twentieth lick. The number of shocks delivered during the 3-min session is recorded for each animal.

EVALUATION

The number of shocks received after treatment is compared with untreated animals. Benzodiazepines increase dose-dependent the number of shocks. Barbiturates in low doses and meprobamate, but not d-amphetamine or scopolamine, are active in this test.

CRITICAL ASSESSMENT OF THE METHOD

The method is far more simple and less time consuming than the methods using conflict behavior after intensive training. The specificity may be less than that of the Geller paradigm.

MODIFICATIONS OF THE METHOD

The method and the apparatus have been modified by Patel and Malick (1982), Patel et al. (1983), Sanger et al. (1985), Langen et al. (2005), and Mathiasen and Mirza (2005).

Miklya and Knoll (1988) showed an increase of sensitivity of the method using rats deprived of food but supplied with tap water ad libitum for 96 h, than fed with dry pellets and punished for drinking during feeding. The punished drinking test has not only be used for identifying and studying anxiolytic agents, but also as a method for measuring anxiogenic activity (Uyeno et al. 1990).

La Marca and Dunn (1994) studied α_2 -antagonists after intravenous administration in the Vogel lick-shock conflict paradigm.

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E.2.4.16**Novelty-Suppressed Feeding****PURPOSE AND RATIONALE**

Placing a hungry rat into an unfamiliar environment with access to food results in a suppression of feeding behavior relative to the condition when the test environment is familiar. This effect has been termed hyponeophagia (Shephard and Broadhurst 1982) and occurs because of the novelty of the test environment. The avoidance of novel foods is termed food neophobia. Both hyponeophagia and food neophobia have been assumed to measure emotionality or anxiety by eliciting a conflict situation arising from a fear of the novel setting and foods, and the drive to eat (Porschel 1971). A number of investigators have adopted these paradigms to explore the behavioral effects of anxiolytics (Soubrie et al. (1975; Cooper and Crummy 1978; Borsini et al. 1993).

PROCEDURE

The testing apparatus consists of individual Plexiglas open fields, 76 × 76 × 46 cm. Thirty Purina lab chow pellets are placed in a pile directly in the center of the open field.

Animals are handled for 3 weeks prior the behavioral testing. Forty-eight hours prior to testing, all food is removed from the home cage, although water is still available ad lib. One h prior to testing, animals receive an intraperitoneal injection of test drugs or vehicle. At the time of testing, the animals are placed into individual open fields containing the food, and the latency to begin eating is measured. If the animal has not eaten within 720 s, the test is terminated and the animal is assigned a latency score of 720 s.

EVALUATION

The data are analyzed by a one-way analysis of variance followed by Fisher Last Significant Difference post hoc tests. An anxiolytic effect is defined as a significant decrease in mean latency to begin eating compared with vehicle controls.

CRITICAL ASSESSMENT OF THE METHOD

The test has the advantage of simplicity for screening procedures.

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E.2.4.17**Shock Probe Conflict Procedure****PURPOSE AND RATIONALE**

The shock probe conflict procedure, an assay responsive to benzodiazepines, barbiturates and related compounds, was described by Meert and Colpaert (1986). Rats being placed in a novel test environment containing a probe, explore the environment and also the probe. The exploration of the probe, quantified as the number of times that the animal makes physical contact with it, is reduced when the probe is electrified. Rats treated with anxiolytics continue to touch the electrified probe.

PROCEDURE

Apparatus: The test environment consists of a Plexiglas chamber, measuring 40 × 40 × 40 cm, and having a metal grid floor. A Teflon probe (∅: 1 cm) with two uninsulated wires (∅: 0.5 mm) each independently wrapped 25 times around it, is inserted from the front panel protruding for a length of 6.5 cm into the test box, 3 cm above the floor of the chamber. The wires are connected to a shocker. Whenever the animal touches both wires simultaneously with some part of its body, a DC current flows through the animal. At the same time, a counter is triggered. Normally, a shock intensity of 0.9 mA is used.

Sixty min after treatment with saline or test substance, the animal is placed in a back corner of the test-box facing away from the probe. The test session starts from the moment makes the first contact and receives the first shock. The number of responses the animal makes during the subsequent 5-min episode is counted.

EVALUATION

Dose-response curves can be established for various drugs at different shock intensities. The Mann-Whitney *U*-test is used to evaluate differences between experimental conditions. To control whether a drug treatment increases responding above the saline control level, an one-tail *t*-test is used; a two-tail test in other cases.

CRITICAL ASSESSMENT OF THE METHOD

The procedure requires neither behavioral training nor expensive equipment and overcomes some of the limitations that are typical for other conflict procedures. However, the procedure still uses electric shock as inhibitory stimulus.

REFERENCES AND FURTHER READING

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barbiturates and related compounds. *Psychopharmacol* 88:445–450

E.2.4.18**Ultrasound Induced Defensive Behavior in Rats****PURPOSE AND RATIONALE**

Rats exposed to aversive stimuli display specific defence behavior as a part of their natural survival strategy. One component of this behavior is the production of ultrasonic calls in the 20–27 kHz range, which are thought to serve a communication role. Artificially generated ultrasound produces intensity-related locomotion, characteristic of defensive behavior (Beckett et al. 1996).

PROCEDURE

The apparatus (Beckett and Marsden 1995) consists of a circular open field arena, 75 cm in diameter, 46 cm high walls, with a video camera suspended above. Locomotor behaviors are recorded and analyzed using a computer automated tracking system capable of following rapid movements (VideoTrack, CPL Systems, Cambridge, UK). This allows the ultrasound-induced change in locomotor behavior to be quantified in maximum speed, average speed and distance traveled by the animals. Data are expressed as 15 sequential 20-s bins over the duration of the experiment.

Ultrasound (continuous tone, square wave, 20 kHz) is produced using a multifunction signal generator at sound pressure intensities of 65, 72 and 75 dB, as measured from the arena, 20 cm horizontally from the speaker. Sound is delivered to the testing arena via a high frequency piezo electric speaker mounted at a height of 40 cm on the wall of the testing arena. The signal frequency and intensity delivered to the speaker are monitored using a digital oscilloscope. White noise is generated using a standard generator and the sound intensity measured as above.

Animals are placed in the test arena 20 min after intraperitoneal injection of drug or vehicle and locomotor activity is measured. After 2 min they are exposed to a 1-min, 20 kHz, square wave ultrasound tone (65, 72 or 75 dB sound pressure intensity, randomized) followed by a further 2 min without sound. This procedure is repeated for each intensity with a 1-min inter-procedure interval. Locomotor activity values are then calculated for the maximum speed, average speed and total distance traveled throughout the 5-min test period and expressed as a series of 15–20-s time epochs.

EVALUATION

Maximum speed is analyzed using a two-way ANOVA. Significant interactions between treatment

and time are followed by one-way ANOVAs for individual time points with post-hoc Duncan's new multiple range test.

MODIFICATIONS OF THE METHOD

Molewijk et al. (1995) evaluated ultrasound vocalizations of adult male rats in association with aversive stimulation as a screening method for anxiolytic drugs.

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E.2.4.19

Anxiety/Defense Test Battery in Rats

PURPOSE AND RATIONALE

Blanchard et al. (1989, 1990, 1992) described a set of procedures designed to assess the defensive reactions of rats to a natural predator, the cat. These tests involve a brief confrontation of laboratory rats with an unconditioned threat stimulus (cat) which, to preclude physical contact, is presented behind a wire mesh barrier. The primary measures, taken both during and after cat presentation, include movement arrest and risk assessment (proxemics/activity test) and the inhibition of non-defensive behaviors (eat/drink or freezing test).

PROCEDURE

The test apparatus for both the proxemics/activity and eat/drink procedures consists of two parallel subject chambers (53 × 20 × 25 cm). The inside walls of each chamber are constructed of opaque black Plexiglas, while outer walls and lids are clear Plexiglas to allow video recording from lateral and overhead views. The end wall of each chamber, constructed of wire mesh, adjoins a separate cat compartment. Subject movements are monitored by five photocells mounted at equal distances over the length of each chamber, and a food hopper and drinkometer are positioned 2.5 cm to each side of the central photocell. Access to the food hopper/drinkometer can be prevented by insertion of Plexiglas gates.

Each rat (Long-Evans strain, female or male, about 100 days old) receives the same injection (drug or saline) in each of the two successive paradigms. The

initial study assesses the effects of cat exposure on proxemics/activity, followed 7 days later by analysis of eat/drink behavior during and after cat exposure. Both procedures are carried out under dim red light.

Proxemic/activity testing. Rats are individually placed in each compartment of the test apparatus. Following a 5-min pre-cat period, the cat is introduced to the cat compartment for 5 min. Following removal of the cat, behavior is recorded for a further 15 min post-cat period, for which measures are summed in three, 5-min blocks. The test session is video recorded for analysis of lying, crouching, rearing, locomotion and grooming. Proxemic location is measured by a digitizing system which divides the length of the subject compartment into thirds, indicating animal's location near the cat compartment, in the midsection of the box, or far from the cat compartment. Assessment of transits indicate movement from one section to another.

Eat/drink testing. Rats are individually given 2 g of finely crushed chocolate cereal on the 2 days after the proxemic/activity test, to familiarize them with this highly preferred food. In order to induce a mild water deprivation, water bottles are removed, 24 h prior to eat/drink testing. On the test day, animals are individually placed in the subject compartments for a 5-min pre-cat period, during which the food hopper and water dispenser are concealed with Plexiglas gates. At the beginning of the 5-min cat period, these gates are removed allowing free access to the crushed chocolate cereal and water. After removal of the cat, the rats are monitored for a further 15-min post-cat period. Measures of eating frequency and duration, and drinking frequency are taken for the cat and post-cat periods.

EVALUATION

The data are analyzed by analysis of variance (ANOVA). Subsequent comparisons between treatment groups and control are carried out using Newman-Keuls procedures.

MODIFICATIONS OF THE TEST

Farook et al. (2001, 2004a, 2004b) and Wang et al. (2003) described a **cat-freezing test apparatus**. The apparatus consisted of a completely enclosed, black, cat compartment (55 × 38 × 30 cm) with a wire mesh floor opening 42 × 19 cm, and an open-top rat compartment (38 × 24 × 19 cm) made of clear Plexiglas to allow observation. The cat compartment could be placed stably on the top of the rat compartment. Male PVG hooded rats weighing 210–260 g were used. Locomotor activity was monitored using an Opto-Varimex Mini (Columbus Instruments), which measured the in-

ruptions of optical beams that were placed 2.5 cm apart. The predator cat was a 4.3-kg male, which was selected for a total absence of aggressive behavior toward the rodents. During a 20-min test period, the duration of freezing, defined as the absence of all movements except movements related to breathing, was monitored. Freezing was expressed as the percentage of time the rat spent frozen during this 20-min session.

Blanchard et al. (1986a, b, 1989) developed a battery of tests designed to elicit a wide range of active and passive defensive activities in **wild rats**.

Griebel et al. (1997, 1998a, 1998b, 2001, 2002) designed a mouse defense test battery in which **Swiss mice** were confronted with a natural threat (a rat) and behaviors associated with this threat were recorded.

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E.2.4.20

Repetitive Transcranial Magnetic Stimulation

PURPOSE AND RATIONALE

Transcranial magnetic stimulation was introduced by Barker et al. (1985). The regional electrical activity in the brain is influenced by a pulsed magnetic field. Repetitive transcranial magnetic stimulation can cause functional changes in the cortex. Effects have been demonstrated in humans as well as in animals (Weissman et al. 1992; Fleischmann et al. 1995; Zyss et al. 1997; Ji et al. 1998; Ben-Shachar et al. 1999; Hausmann et al. 2000; Luft et al. 2001). Most studies indicated an antidepressant or anxiolytic effect (Keck et al. 2000, 2001; Tsutsumi et al. 2002; Kanno et al. 2003); however, this effect is dependent on frequency of stimulation (Sachdev et al. 2002) and duration of treatment (Hedges et al. 2003, 2005; Isogawa et al. 2003). Isogawa et al. (2005) found that 10-day repetitive transcranial magnetic stimulation induced anxiety in normal rats, as evidenced by expression of anxiety behaviors in the elevated plus-maze.

PROCEDURE

Male Wistar rats were used. All animals received repetitive transcranial magnetic stimulation for 10 days. On day 10, groups of 5 rats were treated with saline i.p., or anxiolytics or antidepressants.

Magnetic stimulation was delivered via a high-frequency magnetic stimulator with a round coil (4 cm in diameter) positioned over the rat's head. The stimulator delivered a biphasic cosine current with a pulse width of 200 μ s in duration. The switching elements transfer up to 250 J per pulse to the coil, depending on the intensity setting. The peak magnetic flux at the

center of the coil is approximately 2.2 T at maximal output. The stimulus amplitude was set at an intensity of 1.5 motor thresholds for motor-evoked potentials recorded from the gastrocnemius muscles.

To deliver the stimulation, awake animals were gently held by hand to minimize any discomfort. The coil was held tangentially in direct physical contact with the rat's head. To keep the same brain region stimulated, the center of the coil was set at the center of the vertex of the skull. All animals received one set of training stimuli daily between 13:00 and 18:00 hours for 10 days. To control for the auditory stimulation that accompanies high-frequency magnetic stimulation, sham stimulations were performed with the coil held far enough away (15 cm) from the induced magnetic field.

On the first day of testing, all rats were evaluated on the elevated plus-maze. This consisted of two opposite open arms (50 × 10 cm) without side walls and two opposite enclosed arms (50 × 8 × 40 cm), and was elevated 50 cm above the floor. At the beginning of the test, rats were placed in the middle of the maze facing one of the open arms, immediately left alone in the test room, and were observed for 300 s using a video camera. The following parameters were measured: (1) the time spent in the open arms, (2) total number of entries into the open arms, (3) total number of entries into the closed arms, (4) number of stretched-attend postures, and (5) number of head dips over the edge of the platform.

As a measure of exploratory activity, animals were placed in a standard apparatus. General motor activity was determined by the number of infrared photobeam breaks in a 2-h period. Rats were tested again after treatment with drugs on day 10.

EVALUATION

The results are presented as the means ± SEM of individual values from each group. Overall statistical significance was determined using one-way ANOVA with a post-hoc Dunnett test. For general motor activity, results were analyzed using two-way ANOVA.

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E.2.4.21

Marmoset Human Threat Test

PURPOSE AND RATIONALE

The behavior of the common marmoset (*Callithrix jacchus*) as described by Stevenson and Poole (1976) can

be used for evaluation of potential anxiolytic drugs (Costall et al. 1988).

PROCEDURE

Male or female laboratory bred common marmosets weighing 350–400 g are housed in single sex pairs. Holding rooms are maintained at 25°C at a humidity of 55% on a 12 h light/dark cycle. Tests are conducted between 13:30 and 15:30 in the normal holding room. The holding cages are 75 cm high, 50 cm wide and 60 cm deep.

A behavioral change characterized by retreat from, and posturing toward a human threat is initiated by a human observer standing in close proximity in front of the holding cage. Changed behavior is recorded over a 2 min period by the observer. The behavioral measures selected are:

- i. the % of time spent on the cage front in direct confrontation with the human threat,
- ii. the number of body postures, primarily shown as raising the tail to expose the genital region with varying degrees of piloerection, anal scent marking and slit stare with flattened ear tufts.

The animals are used at 7 day intervals and are subject to a random cross-over of treatments.

Drugs are administered 45 min before exposure to the human threat situation.

EVALUATION

Statistical analysis is performed with one-way analysis of variance followed by Dunnett's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The human threat test in marmosets has the advantage of using primates instead of rodents. However, it is subject to individual scoring of the observer.

MODIFICATIONS OF THE TEST

Cilia and Piper (1997) developed a method of measuring conspecific confrontation-induced behavioral changes in common marmosets together with automated monitoring of locomotor activity as a possible model of anxiety.

Barros et al. (2000) measured fear and anxiety in the marmoset (*Callithrix penicillata*) with a novel predator confrontation model. The wild cat (*Felis tigrina*) was chosen as taxidermized predator to induce anxiety-related behaviors.

Borsini et al. (1993) used female **cynomolgus monkeys** to test aggressiveness against the observer.

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E.2.4.22

Psychosocial Stress in Tree Shrews

PURPOSE AND RATIONALE

Male tree shrews (*Tupaia belangeri*) provide an animal model to study the neurobehavioral and endocrine consequences of chronic psychosocial stress (Fischer et al. 1985; Fuchs et al. 1993, 1996; Fuchs and Flügge 2002; Fuchs 2005). When living in visual and olfactory contact with a male conspecific by which it has been defeated, the subordinate tree shrew shows dramatic behavioral, physiological, and neuroendocrine changes. The pattern of these changes resembles a depression-like symptomatology.

PROCEDURE

Animals are subjected to a 10-day period of psychosocial conflict to elicit stress-induced behavioral and endocrine alterations before the onset of drug treatment. For a treatment period of 30 days, animals are given either saline or an oral dose of test compound.

EVALUATION

At the end of the test period, the following parameters are recorded: behavioral changes (marking, groom-

ing, locomotor activity) and endocrine changes (urinary cortisol and norepinephrine excretion).

MODIFICATIONS OF THE METHOD

Lucassen et al. (2004) found that treatment with an antidepressant reduces apoptosis in the hippocampal dentate gyrus and temporal cortex induced by a 7-day stress period in tree shrews.

Czéh et al. (2005) examined the effects of a NK₁ receptor antagonist in the chronic psychological stress model of adult male tree shrews.

Shively et al. (2005) described social stress-associated depression in adult female *cynomolgus monkeys*.

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E.2.4.23

Aversive Brain Stimulation

PURPOSE AND RATIONALE

Electrical stimulation of brain aversive areas, in particular the midbrain central gray, induces defensive reaction and/or flight behavior in several species and, therefore, may be viewed as an animal model of anxiety or of panic attack. Most studies used intracerebral microinjections of neurotransmitters, their agonists and antagonists to elucidate the mechanisms of aversive or antiaversive effects (Schütz et al. 1985; Graeff et al. 1986, 1990, 1991, 1993, 1997; Audi et al.

1988, 1991; Brandão et al. 1991, 1993; Broekkamp et al. 1991; Nogueira and Graeff 1991, 1995; Motta and Brandão 1993; Aguiar and Brandão 1994, 1996; Melo and Brandão 1995; Motta et al. 1995; De Araujo et al. 1998). Other groups evaluated the effect of drugs, e. g., serotonin receptor antagonists, on periaqueductal gray stimulation induced aversion after peripheral application (Bovier et al. 1982; Clarke and File 1982; Jenck et al. 1989, 1995, 1996, 1998, 1999; Beckett and Marsden 1997). Dorsal periaqueductal gray-induced aversion is considered as a model of panic anxiety.

PROCEDURE

Surgery. A stainless steel bipolar twisted electrode, insulated to the tip which is cut square to the shaft, is implanted to the dorsal part of the periaqueductal gray matter of male Wistar rats weighing 370–450 g under pentobarbital anesthesia. According to the atlas of Paxinos and Watson (1982), the coordinates for the electrode tip are 5.8 mm posterior to the bregma, 0.2 mm lateral to the midline and 5.0 mm ventral to the surface of the skull. The electrode is held in place with dental cement and five screws threaded into the skull.

Behavioral Procedure. Animals are placed in a rectangular cage (20 × 36 × 20 cm high) with a grid floor and a 2 cm high barrier dividing the cage in half. They are allowed to explore freely for 10–15 min before the stimulation begins.

Brain stimulation consists of constant current square wave, 0.1 ms duration, monophasic pulses conducted from the neurostimulator (Grass S88 + stimulus isolation unit SIU8) to the electrode by way of flexible wire leads. Pulse duration, pulse frequency and stimulation intensity are monitored by an oscilloscope. Animals are screened for stimulation-induced aversion using a fixed stimulation frequency of 50 Hz; current is raised slowly until aversive behavioral signs are observed. Aversive effects are first characterized by visible autonomic reactions (increase in respiratory rates, piloerection, eventually mydriasis) in animals which are behaviorally frozen. Increasing the intensity induces, following a freezing period, active behavioral signs, ranging from ear dressing and head weaving to sudden running and attempts to escape out of the cage.

With this fixed stimulation intensity, aversive behavior is shaped into an operant escape response: rats are trained to stop the stimulation by escaping from one compartment to the opposite compartment of the cage. Brain stimulation is switched off when the rat crosses the middle line separating the two compartments or after a maximal cut-off time of 20 s. A trial is

applied every min. Three to 10 daily sessions of 30 trials are required to obtain stable responses.

Self-Interruption Threshold Determination

Once an animal displays steady performances during 3 consecutive days on this task with fixed intensity and fixed stimulation frequency, it undergoes the next step of training aimed at determining its stimulation frequency threshold for escape reaction. This consists of testing the animals in a threshold procedure in which the frequency is varied while stimulation intensity is held constant. This procedure keeps the size of the stimulation field around the electrode tip constant.

A stimulation intensity is chosen and defined as the threshold intensity eliciting escape when the stimulation frequency is 50 Hz. With this intensity held fixed, a method of limits is employed to determine the frequency threshold for escape: the stimulation frequency is either decreased or increased depending on the response displayed by the animal on the previous trial. Starting from 50 Hz, stimulation frequency is decreased by 5 Hz steps following a trial in which the rat responds to the stimulation, and is increased by 5 Hz steps, in case the animal fails to respond to the stimulation. When a response is made, the time elapsing between the onset of the stimulation and the moment the animal crosses the midline barrier is also recorded as escape latency; no response is associated with the maximum cut-off time of 20 s.

Frequency threshold is calculated as the average frequency eliciting an escape reaction during a 20-min pre-injection session; an average escape latency is calculated the same way. Drugs are then injected intraperitoneally at various doses and 35 min following administration, frequency threshold is determined again over a 25–30 min post-injection session. Drug effects can be estimated by comparing thresholds and latencies for each animal before and after injection. Doses are injected at least 4 days in a counterbalanced order. Animals serve as their own controls and can undergo several treatments.

EVALUATION

Data are analyzed by means of analysis of variance, followed by paired *t*-tests. Dose-response curves are established for active drugs.

MODIFICATIONS OF THE METHOD

The group of Graeff and Brandão (1986) used an electrode-cannula, called chemitrode, for electrical stimulation and microinjection of drugs or neurotransmitters at the same place of the periaqueductal gray. This

chemitrode was made of stainless steel cannula (outside diameter 0.6 mm, length 12.5 mm) glued to a brain electrode made of stainless steel (diameter 250 mm) enamel insulated except at the cross-section of the tip, reaching 1 mm below the lower end of the cannula (Nogueira and Graeff 1995).

Schenberg et al. (2001) appraised the isomorphism of dorsal periaqueductal gray-evoked defensive behaviors and panic attacks both in rats and humans.

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E.2.5

Conditioned Behavioral Responses

E.2.5.1

Sidman Avoidance Paradigm

PURPOSE AND RATIONALE

Sidman (1953) described an apparatus for the evaluation of two temporal parameters (shock-shock interval and response-shock interval) of the maintenance of avoidance behavior by the white rat. The procedure

has been widely used to evaluate CNS depressant compounds, neuroleptics, anxiolytics and sedatives.

PROCEDURE

The test cage is equipped with a single lever and a light. This cage is enclosed in a sound-attenuating chamber with a fan and with a speaker emitting a white-noise auditory background. The test cage has a grid floor of steel bars which are attached to a scrambled shock source. The data are recorded in an adjacent room. Male Sprague-Dawley rats with a starting weight of 250 to 300 g are housed in individual cages. They are trained to avoid an unsignalled shock by repetitive lever-pressing responses. A shock (1.5 mA for 0.5 s) is delivered to the grid floor every 15 s if no responses occur (shock-shock interval of 15 s = SS-15 s). A lever press (response) will delay the oncoming shock for 30 s (response-shock interval of 30 s = RS-30 s). The responses do not accumulate for delays of shock; a shock will be delivered 30 s after the last response is made even if 10 responses are made 31 s prior. Every 30 min, the total number of shocks received and the total number of responses made are accumulated and constitute the basic data. The animals are trained until they maintain a stable response rate and receive no more than 100 shocks/five hour test session. After reaching these criteria of performance, experimental compounds are administered and their effects on the performance of this learned avoidance behavior are evaluated. The experimental compounds or the standard are usually administered by i.p. injection immediately prior to testing in volumes of 1 ml/kg of body weight. Depressant drugs lower the rate of lever presses and increase the number of shocks received. Stimulant drugs increase the rate of lever pressing.

EVALUATION

The effect of a drug on the performance of an animal is compared to the data generated in the previous non-drug sessions. Each animal thereby serves as its own control. The basic measures of performance during a specific time interval, responses and shocks are used for evaluation. Responses are reported both as total and as percent of control responses. Shocks are reported as totals and as shock-avoided (SHA) as percent of control. This latter measure is computed by subtracting the number of shocks received from the total number of possible shocks if no responses had been made. In the initial screening of experimental compounds, the results are reported in terms of the total effect during a five-hour test. However, an *ED*₅₀ is usually estimated during a representative time of peak activity.

CRITICAL ASSESSMENT OF THE METHOD

Anxiolytics show activity in this test, however, it has been proven to be more reliable for neuroleptic activity. Apparently, the present conditioned active avoidance paradigms do not constitute a reliable method for screening anxiolytic agents, in spite of their homologies with human anxiety.

MODIFICATIONS OF THE METHOD

Heise and Boff (1962) and Galizio et al. (1990) extended the method by using two levers, an “avoidance lever” and an “escape lever” for calculating ratios between shock rate, escape failure rate and avoidance rate.

Balfour (1990) described the effect of drugs on rat behavior in an unsignalled Sidman avoidance schedule.

Wadenberg et al. (1998) described and evaluated a newly designed apparatus for the assessment of conditioned avoidance response performance in rats.

Patel and Migler (1982) reported a sensitive and selective conflict test in **squirrel monkeys**.

Szewczak et al. (1995) tested antipsychotic agents using continuous avoidance behavior in adult male squirrel monkeys.

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E.2.5.2

Geller Conflict Paradigm

PURPOSE AND RATIONALE

Experimentally induced conflict by punishing food-rewarded behavior has been used to differentiate between various psychoactive drugs by Geller and Seifter (1960). The basic principle has been used and modified by many authors to reveal possible anti-anxiety effects of experimental compounds.

PROCEDURE

Male albino rats with a starting body weight of 300–400 g are housed individually. They are food deprived until the body weight is gradually reduced by approximately 20% of original and it is maintained at this level by restricted food diet. Conditioning is carried out in commercially available Skinner boxes (e.g. Campden Instruments, London, UK) equipped with a house light, a single lever, cue lights, a liquid dripper, and a grid-floor connected to a shocker. Sweetened condensed milk delivered by the liquid dipper serves as the positive reinforcer. The data are recorded on cumulative recorders.

The animals are trained to lever press for the milk reward in two distinct response-reward sections. In the anxiety or “conflict” segment (signalled by onset of both tone and cue lights), a dipper of milk is delivered in response to each lever press (continuous reinforcement schedule = CRF) However, lever presses during this period are also accompanied by a 40-ms pulse of aversive foot-shock through the grid floor. This creates a conflict between milk reward and the a painful foot shock. This conflict period is 3 min in duration.

During the other segment of this paradigm, the lever presses produce a drop of milk only at variable intervals of time from 60 to 210 s with an average reward of once per 2 min (variable interval = VI-2 min). No shocks are administered during this variable interval phase of testing which is 15 min in duration.

The test procedure consists of four 15 min non-shock variable interval segments where reinforcement is available on a restricted basis. Each variable interval period is followed by a three minute CRF-conflict period phase when reinforcement is constantly available but always accompanied by an aversive footshock. The shock level is adjusted for each subject to reduce the CRF responding to a total of less than 10 lever presses during the entire test. The rats are tested 2 to 4 days a week. Drugs are administered once per week and the performance is compared to the previous day’s control trial. The VI responses are used to evaluate any general

debilitating drug effects while the CRF responses are used to evaluate any anti-anxiety effects as indicated by the increased responding during the CRF conflict period.

The test compounds are administered intraperitoneally 30 min or orally 60 min before the test period.

EVALUATION

The total number of lever presses during the conflict periods (CRF) and the non-conflict periods (VI) are counted. Values of treatment sessions are expressed as percentage of values of the preceding non-treatment day. An increase of lever presses in the conflict period is regarded as indication of an anti-anxiety effect, and a decrease of lever presses in the non-conflict period as an indication for a sedative effect. In this procedure linear dose-response curves are rarely found. Therefore, minimal effective doses (MED) are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method is suitable to distinguish anxiolytics from other centrally active drugs, such as sedatives and neuroleptics. The relative potency of various anxiolytic agents in a number of species compares favorably with their relative potency in humans. The negative aspects are the time consuming procedure, the high expenses for the apparatus and the difficulty to obtain ED_{50} -values.

The relevance of this test and other models has been challenged by Bignami (1988).

MODIFICATIONS OF THE METHOD

The method has been modified by many authors.

Davidson and Cook (1969), Cook and Davidson (1973) introduced a schedule of 10 lever presses for the delivery of one food pellet together with an electric foot-shock. In the variable interval reinforcement period, responses were reinforced by food delivery at varying intervals of time; the mean interval was 30 s. Also under these conditions differentiation of various psychotropic drugs could be achieved.

Iorio et al. (1986) and Chipkin et al. (1988) used a procedure during which a tone (5 s) preceded and then overlapped for 10 s with scrambled footshocks. After the start of the tone, the rat has the option to avoid (in the no shock period) or escape (in the shock period) the shock by jumping onto a platform located 17 cm above the grid floor.

Thiébot et al. (1991) developed the method further by introducing "safety signal withdrawal", a behavioral paradigm in rats sensitive to both anxiolytic and anxiogenic drugs.

Commissaris and Fontana (1991) published a potential animal model for the study of antipanic treatments. Rats were trained to drink their daily water ration during 10-minute sessions. These sessions were characterized by alterations of silence (unpunished periods) and the presence of a tone (punished periods). Tube contact during the tone periods resulted in a 0.25–0.5 mA shock delivered to the mouth of the animal for the duration of the tube contact. After 4 weeks of training, subjects received either chronic post-test treatment with an antidepressant or vehicle, twice daily, seven days per week. Acute pre-test administration of traditional anxiolytics (benzodiazepines, barbiturates) increased dramatically the number of punished contacts made in a dose-dependent manner. Typical antidepressants were not active after pre-test administration, however, active after chronic post-test treatment.

Beaufour et al. (1999) investigated the effects of chronic antidepressants in the conditioned suppression of operant behavior in rats. In daily 18-min sessions, three periods of non-punished lever pressing for food alternated with two 4-min periods signaled by a light-on conditioned stimulus during which 50% of the responses were randomly punished by electric foot shocks. Antidepressants were administered once daily for 7–8 weeks to trained, food-restricted rats.

The conflict test has also been adapted to the **mouse** (Prado de Carvalho et al. 1986).

The conflict behavior and anticonflict effect of anxiolytics has been demonstrated in a variety of species, including **pigeons** (Morse 1964; Wuttke and Kelleher 1970; McMillan 1973; Gleeson et al. 1989; Barrett et al. 1989; Barrett 1991; Schipper et al. 1991; Pollard et al. 1992; Barrett et al. 1994; Mos et al. 1997).

Patel and Migler (1982) described a sensitive and selective conflict model in male squirrel **monkeys** in which anti-anxiety agents exhibit pronounced anticonflict activity.

Ervin et al. (1987), Ervin and Cooper (1988), van Heest et al. 1991; Simiand et al. (1993) used **conditioned taste aversion** as a conflict model. Moderate taste aversions were induced by pairing the initial consumption of 0.25% sodium saccharin with either 25 mg/kg 5-hydroxytryptophan or 30 mg/kg i.p. LiCl. Antagonism was found with benzodiazepines and non-benzodiazepine-anxiolytic drugs.

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E.2.5.3**Progressive Ratio Procedure****PURPOSE AND RATIONALE**

The progressive ratio schedule was introduced as a model for studying the psychomotor stimulant activity of drugs in the rat by Poncelet et al. (1983). Male Wistar rats were trained to press a lever with food reinforcement according to a continuously reinforced schedule (CRF). Afterwards, rats were subjected to three experimental sessions (30 min each) during which responding was rewarded according to a progressive ratio schedule (following an initial 2-min CRF period, the number of presses necessary for the pellet delivery was doubled every second minute). Responding during the first half of each session, i. e., pressing for food, was maintained at a significant level, whereas it was almost suppressed during the second part of the session. Animals treated with stimulants showed an increased rate of responding.

Several authors used the progressive ratio test (Richardson and Roberts 1991; McGregor et al. 1993; Bourland and Frenche 1995; Ferguson and Paule 1996; Pulvirenti et al. 1997, 1998; Duvauchelle et al. 1998; Grottick et al. 2000; Mobini et al. 2000; Wilcox et al. 2000; Woolverton et al. 2002; Schneider und Koch 2002,2003; Kozinowski et al. 2003; Solinas et al. 2004).

PROCEDURE

Drews et al. (2005) studied the effects of a cannabinoid agonist on operant behavior and locomotor activity in rats. The progressive ratio test was conducted in an operant chamber (24 × 28 × 28 cm) (Operant Behavior System, TSE Systems, Bad Homburg, Germany). First, male Wistar rats were habituated for 1 day to the test chamber, the palatable casein pellets and the noise of the magazine response. After shaping, rats were trained over 3 days at lever pressing in sessions of 30 min on a continuous reinforcement schedule until they reached a stable baseline. After lever pressing was completed, one progressive ratio test session (for 30 min) was conducted on the next day. The progressive ratio schedule (i. e., lever presses for one pellet) was changed every second minute according to the following exponential progression: 1, 2, 4, 6, 9, 12, 15, . . . , derived from the formula $5 \cdot e^{0.2n} - 5$, where n is the position in the sequence of ratios (Mobini et al. 2000). The so-called break point, the conventional index of performance on a progressive ratio schedule of reinforcement (Reilly 1999), was defined as the first progressive ratio sequence where lever pressing de-

creased $\leq 50\%$ relative to the previous phase without increasing $\geq 100\%$ in the following phase.

EVALUATION

Student's t -test was used to evaluate operant responding in the progressive ratio test.

MODIFICATIONS OF THE METHOD

Barr and Phillips (1999) found that withdrawal following exposure to d-amphetamine decreases responding for a sucrose solution as measured by a progressive ratio schedule of reinforcement. The authors concluded that the progressive ratio procedure may be a useful technique for evaluating changes in motivation to natural reinforcing stimuli following withdrawal from psychostimulant drugs.

For safety studies, the response requirement increases for every infusion or after a fixed period of time until the animal ceases to lever-press for a pre-determined interval, defined as the breaking point. The presumption of progressive ratio studies is that the higher the breaking point the greater the reinforcing effectiveness, although the same potential complications regarding pharmacokinetics that were noted above for a simple (FR) schedule of self administration also apply to progressive ratio schedules.

Weed et al. (1997) studied the relationship between reinforcing effects and *in vitro* effects of D1 agonists in **rhesus monkeys**.

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E.2.5.4

Conditioned Defensive Burying in Rats

PURPOSE AND RATIONALE

Besides the well known defensive reactions of animals in typical laboratory settings, like freezing, fleeing, and attacking, rats tested in the presence of bedding mate-

rial develop a peculiar behavior when shocked through a stationary prod by burying the shock source. This behavior has been proposed as a new paradigm for the study of anxiolytic agents (Pinel and Treit 1978, 1983; Treit et al. 1981).

PROCEDURE

Male adult rats weighing 250–400 g are used. The testing is performed in 44 × 30 × 44 cm Plexiglas test chambers, the floor of which is covered with 5 cm of a commercial bedding material. In the center of each of the four walls, 2 cm above the level of the bedding material, is a small hole through which a 6.5 × 0.5 × 0.5 cm wire-wrapped wooden dowel (i. e., the shock prod) can be inserted. Electric current is administered through the two uninsulated wires wrapped around the prod. The behavior of each rat is monitored for 15 min from a separate room via closed circuit television.

Before each of the experiments, the rats are placed in the Plexiglas test chamber in groups of 5 or 6 for 30-min periods on each of 4 consecutive days. In the experiments, groups of 10 animals are used for each dose and control. The rat is injected intraperitoneally with the test drug or saline before being placed into the test chamber in which the shock prod is inserted. The animal is placed into the center of the chamber so that it faces away from the prod. When the rat first touches the prod with its forepaw, it receives a brief electric shock (1 mA) which typically elicits a flinch away from the prod and withdrawal towards the back of the chamber. Afterwards, the rat moves directly towards the prod, pushing and spraying a pile of bedding material ahead with rapid shoveling movements of its snout and alternating pushing movements of its forepaws. The prod is buried in a pile of bedding material. The duration of burying is recorded.

EVALUATION

The mean duration of burying in treated animals is compared with controls. Anxiolytics as well as neuroleptics shorten the burying behavior dose-dependent at low shock intensity (1 mA). A second measure is the height of the bedding material. At high shock intensity (10 mA) only neuroleptics but not benzodiazepines are active.

CRITICAL ASSESSMENT OF THE METHOD

The method of conditioned defensive behavior can be regarded as a simple and reliable method to detect anxiolytic activity of benzodiazepines and neuroleptics. However, using higher shock intensity – where only

chlorpromazine was found to be active – a separation between benzodiazepines and neuroleptics has been demonstrated.

The drug-class specificity of the test has been challenged by Craft et al. (1988).

MODIFICATIONS OF THE METHOD

Diamant et al. (1991) used telemetry to register autonomic and behavioral responses in the shock-prod burying test in rats.

Wiersma et al. (1996) reported that microinfusion of corticotropin-releasing hormone in the central amygdala of freely moving rats enhanced the active behavior responses in the conditioned defensive burying paradigm.

Fernandez-Guasti and Lopez-Rubalcava (1998) used the rat burying behavior test to study the effect of various potential anxiolytics.

Broekkamp et al. (1986), Njung'e and Handley (1991a, b; Gacsályi et al. 1997) described burying of marbles by *mice* as harmless objects without punishment by electrical shocks as a model for detection of anxiolytics. Thirty min after treatment with drugs, male Swiss mice, weighing 20–24 g, were individually placed in a 23 × 17 × 14 cm cage with 25 glass marbles, 1.5 cm in diameter. The glass marbles were placed in close contact in the middle of the cage on a 5 cm layer of sawdust. The mice were left in the cage with the marbles for 30 min after which the test was terminated by removing the mice and counting the number of marbles that were more than two thirds covered with sawdust.

DeBoer and Koolhaas (2003) reviewed the ethology, neurobiology and psychopharmacology of defensive burying in rodents.

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E.2.5.5

Taste Aversion Paradigm

PURPOSE AND RATIONALE

When ingestion of a taste stimulus is paired with internal malaise the animal remembers the taste and rejects its ingestion thereafter. This phenomenon is called a conditioned taste aversion or taste aversion learning. In the classical experiment in rats, the conditioned stimulus is a 0.01 M drinking solution of saccharin paired with an intraperitoneal injection of 0.15 M LiCl solution as unconditioned stimulus. The underlying neural mechanisms (Yamamoto 1993; Agüero et al. 1993, 1996; Swank et al. 1995) as well as facilitating and inhibiting factors (Lipinski et al. 1995; Sobel et al. 1995) were investigated. Moreover, drugs by themselves can be studied. Rats are presented a fluid with palatable taste and immediately after consumption of this liquid injected with a drug, whose effect the animals have not experienced before. Subsequently, on a later occasion and under non-drug conditions, avoid-

ance of the taste associated with the drug is measured (De Beun et al. 1996).

PROCEDURE

Male Wistar rats weighing 220–250 g are housed in groups of four per cage under a normal 12L:12D regime at 22–23°C with free access to food and water. Twenty-four hours before the first conditioned taste aversion session, the animals are water deprived and fluid access is from then on restricted to daily experimental sessions of 15 min which takes place individually in a test cage. After each session, the animals are returned to their home cages. Food is freely available in the home cages throughout the procedure, but not available during the sessions. For a given subject, all six sessions required to complete a conditioned taste aversion experiment take place in the same test cage. Animals designated to the same experimental group are run in parallel. During the first four sessions (day 1 through day 4), both bottles contain plain tap water. During this phase of the procedure, the animal learns to drink a reasonable amount of fluid in a short period of time. For the 5th session (day 5, conditioning session), both bottles are filled with a 0.1% saccharin solution and immediately after completion of this session the animals are injected with either the vehicle or different doses of the test drug. Per animal, only one dose (or the vehicle) of a particular drug is tested. On days 6 and 7, no sessions are conducted (washout period) and the animals have free access to tap water in the home cages from the end of day 5 until the morning of day 7, when the animals are again deprived of water, 24 h prior to the final 6th session (day 8, test session). During this last session, one bottle contains the saccharin solution and the other bottle is filled with tap water. To control for location bias, the saccharin is presented in the left bottle for half of the animals in each group and in the right bottle for the other half. By measuring the amount of fluid consumed from both bottles separately, drug-induced conditioned taste aversion can be determined by comparison of the relative saccharin intake in the drug treated groups and their vehicle-treated controls.

EVALUATION

Data of test drugs are submitted separately to one-way analysis of variance, with the between-subjects factor DOSE. The dependent variable is the ratio of (saccharin solution/saccharin solution+tap water) intake. Fluid intake scores are calculated in grams. Post hoc analyses are used with Tukey HSD multiple comparisons. Results are considered significant when $p < 0.05$.

MODIFICATIONS OF THE METHOD

Besides LiCl, several other drugs were used to induce taste aversion, such as ethanol (Gauvin and Holmway 1992; June et al. 1992; Thiele et al. 1996; Bienkowski et al. 1997), morphine (Miller et al. 1990; Bardo and Valone 1994), cocaine (Van Haaren and Hughes 1990; Glowa et al. 1994), naloxone (Mucha 1997), apomorphine (McAllister and Pratt (1998), caffeine (Brockwell et al. 1991), d-amphetamine (Davies and Wellman 1990; Lin et al. 1994), nicotine (Shoabit and Stolermmann 1996), quinine (Parker 1994), cisplatin (Mele et al. (1992), benzodiazepine and non-benzodiazepine anxiolytics (Neisewander et al. 1990), 5-hydroxytryptamine (Rudd et al. 1998), dopamine D₃ agonists (Bevins et al. 1996), cyclosporine A (Exton et al. 1998), cholecystokinin (Ervin et al. 1995; Mosher et al. 1996) and Δ^9 -tetrahydrocannabinol (Parker and Gillies 1995).

Turenne et al. (1996) found individual differences in reactivity to the aversive properties of drugs. Rats were assigned to high conditioned taste avoidance and low conditioned taste avoidance groups on the basis of their intake of saccharin solution previously paired with morphine, amphetamine, lithium, or fenfluramine.

Willner et al. (1992) investigated the influence of drugs on taste-potentiated odor aversion learning in rats.

Rabin and Hunt (1992) studied taste aversion learning in **ferrets**.

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E.2.5.6

Cued and Contextual Fear Conditioning

PURPOSE AND RATIONALE

Fear-conditioning is a form of Pavlovian conditioning where an animal is trained to associate a neural stimulus (e. g., a 10-s presentation of light) with an aversive, unconditioned stimulus (US), such as an electric foot shock. After such pairings, the light alone predicts the occurrence of the shock and acts as a conditioned stimulus (CS), eliciting a state of fear. Tones, lights, odors and tactile stimuli have been used as CS in fear-conditioning experiments (Anagnostaras et al. 1999; Fendt and Fanselow 1999). A startle response is elicited by a sudden acoustic, visual or tactile stimulus and is composed of a fast, sequential muscle contraction, with the most prominent reaction around the face, neck, and shoulders.

The **fear-potentiated startle paradigm** was initially described by Brown et al. (1951). Rats are given several pairings of light CS and foot shock. After this procedure, the mean amplitude of the acoustic startle response to a loud noise is usually 50%–100% higher in the presence of the light CS than to the noise alone. The difference between these two trial types represents

the fear potentiation of the startle response and acts as a measure of fear.

In the **cued and contextual fear conditioning paradigm**, animals learn to associate a novel environment (context) and a previously neutral stimulus (conditioned stimulus, a tone) with an aversive foot shock stimulus. Testing then occurs in the absence of the aversive stimulus. Conditioned animals, when exposed to the conditioned stimuli, tend to refrain from all but respiratory movement. Freezing responses can be triggered by exposure to either the context in which the shock was received (context test) or the conditioned stimulus.

Sullivan et al. (2003) determined the effects of doxapram for cue and contextual fear conditioning in rats.

PROCEDURE

Fear Conditioning Apparatus

Pavlovian fear conditioning was performed using a clear polycarbonate conditioning chamber encased by a sound-attenuating outer box and fitted with metal bars as a floor that can deliver current (context 1). For fear conditioning to cue, the context was changed between the conditioning and the testing day to enhance the specificity of the fear response to the tone cue on testing. For the testing day, striped walls were placed on the outer surface of the clear chamber walls, the floor was fitted with a black panel covering the bars, the ambient light was lower, and the floor had a peppermint odor from cleaning with a scented detergent (context 2). A microcamera on the ceiling of the chamber allowed observation during the experiments and a videotape record for scoring of behavior.

Cued fear conditioning behavioral procedure

On conditioning day 0, adult male Sprague Dawley rats were habituated to the conditioning chamber for 15 min. On conditioning day 1 the rats received an intraperitoneal injection of drug and were returned to the home cages. After 10 min each rat was placed in the conditioning chamber (context 1) and was trained with two trials of tone-shock pairings. The tone (10 kHz, 72 dB) lasted 20 s and co-terminated with a 0.5-s, 7-mA shock. The intertrial interval was variable (mean 120 s) and was generated by a computer program. Videotape of the conditioning allowed later scoring of the level of freezing during each tone. On test days 1, 2, and 3, rats were tested with the different contextual stimuli in the chamber (context 2). On each day they received ten presentations of the 20-s tone

(no shocks) while being videotaped for later scoring of the time spent freezing during each tone. Rats were matched by weight for control and various doses of test drug.

Contextual fear-conditioning behavioral procedure

On conditioning day 0, the rats were habituated to the conditioning chamber (context 1) for 20 min. On conditioning day 1, the rats received an intraperitoneal injection of drug and were returned to their home cages. After 7 min, each rat was placed in the same chamber (context 1), and at 8 min after injection the computer program was initiated that trained with two shocks separated by a variable intertrial interval (mean 120 s). The shocks were 1.5 mA and lasted 0.5 s, and the total time in the chamber was 8 min for all subjects. On day 2, rats were tested in the same chamber (context 1) for 5 min while being videotaped for later scoring. The 30-s periods before and after shock 1, the 30-s period after shock 2, and the first 30 s of each minute during the 5 min of testing were scored for freezing. Rats were matched by weight for vehicle and drug-treated groups.

EVALUATION

Data were analyzed with a series of one-way analyses of variance with repeated measures or with Student's *t*-tests.

MODIFICATIONS OF THE METHOD

Several modifications of this procedure have been used for various purposes (Phillips and LeDoux 1992; Lu and Wehner 1997; Maren 1998; Laurent-Demir and Jaffard 2000; DeLorey et al. 2001; Gupta et al. 2001; Malkani and Rosen 2001; McKay et al. 2002; Riedel et al. 2002; Cambon et al. 2003; Fischer et al. 2003; Maciejak et al. 2003; Walker and Carrive 2003; Sienkiewicz-Jarosz et al. 2003; Célérier et al. 2004; Kudo et al. 2004; Mesches et al. 2004; Roberts et al. 2004; Takahashi 2004; Wehner et al. 2004).

Zhang et al. (2004) used a telemetric system for recording cardiovascular data and automated measurement of conditioned freezing behavior.

Contarino et al. (2002) described automated assessment of conditioning parameters for context and cued fear in mice.

Several authors (Radulovic et al. 1998; Stiedl et al. 1999, 2000; Kishimoto et al. 2000; Eckart et al. 2001; Maciejak et al. 2003; Tezval et al. 2004; Vöikar et al. 2004; Radyushkin et al. 2005; Tovote et al. 2005) used a commercially available fear-conditioning system (TSE Systems, Bad Homburg, Germany) consist-

ing of the following components: up to four boxes with animal location sensors, shock grid and test arena; box housing with software-controlled loud speaker, software-controlled light and ventilator; control unit with integrated shocker/scrambler; fear-conditioning software; PC-interface.

Crestani et al. (2002) and Gould et al. (2004) distinguished between delay fear conditioning and trace fear conditioning. For delay fear conditioning, after 3 min exposure to the chamber mice received three successive tone-shock pairings with the shock delivered during the last 500 ms of the tone. Freezing was recorded 48 h later in a modified context (new olfactory, tactile, and visual cues) for 3 min and subsequently in the presence of the tone for 8 min. For trace fear conditioning, the procedure was similar to that for delay conditioning, except that an empty trace interval of 1 s was interposed between the tone and the foot shock in three learning trials.

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E.2.6

Effects on the Endocrine System

E.2.6.1

Plasma Catecholamine Levels During and After Stress

PURPOSE AND RATIONALE

A wide variety of stressors causes significant biochemical, physiological, and behavioral changes. These changes include marked increases in plasma catecholamines, heart rate and blood pressure. Treatment with anxiolytic drugs can attenuate the stress induced increases of norepinephrine and epinephrine in plasma (Vogel et al. 1984; Livesey et al. 1985; Taylor et al. 1989; de Boer et al. 1990; Krieman et al. 1992).

PROCEDURE

Male Sprague-Dawley rats, 300–350 g, are individually housed and given food and water ad libitum. After 1 week of acclimatization, an aortic catheter is surgically implanted in each animal, running on top of the psoas muscle and brought out through an incision at the back of the neck. The catheters are flushed daily with heparinized saline. After a recovery period of 48 h baseline (time –15) blood samples are drawn and blood pressure recordings are taken using a Grass model 7 polygraph. The animals are then given an i.p. injection of the test compound or the vehicle. 15 min after the injection (time 0), blood samples, and heart rate and blood pressure measurements are taken. Animals in control and treatment groups are then stressed by immobilization for 1 h and blood samples, heart rate and blood pressure recordings are taken at time 15, 30 and 60 min during stress. Immobilization is performed by taping the legs of the animals to the laboratory bench. After the stress period of 1 h, the animals are released, returned to the home cage for recovery and 1 h post-stress samples and recordings are taken. Non-stressed, compound or vehicle treated animals remain in the home cage for the entire test period and are sampled in the same manner as the stressed animals. Approximately 0.3 ml blood is withdrawn for each sample and an equal amount of 0.9% saline is re-infused to prevent changes in blood volume.

EVALUATION

Determinations of norepinephrine and epinephrine in plasma are made using a radio-enzymatic assay. Heart rate and systolic and diastolic blood pressure are determined directly from the polygraph tracings. The data are analyzed using a three-way analysis of variance (ANOVA) with repeated measures, two-way ANOVA, Student's *t*-test, Student-Newman-Keuls and the trapezoidal rule for the area under the curve.

CRITICAL ASSESSMENT OF THE METHOD

Reduction of catecholamine levels but no changes of the cardiovascular parameters could be found after treatment with anxiolytics. The test can be used as a method for evaluation of the influence of psychoactive drugs on the endocrine system (Krieman et al. 1992).

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E.2.6.2

Plasma Corticosterone Levels Influenced by Psychotropic Drugs

PURPOSE AND RATIONALE

Corticosterone levels in the blood of rats are elevated not only after stress but also after application of selective 5-HT receptor agonists (Koenig et al. 1987). This has been used to differentiate typical and atypical neuroleptics (Nash et al. 1988).

PROCEDURE

Male Sprague Dawley rats weighing 200–250 g are housed 6 per cage under temperature and light/dark controlled conditions with free access to food and water. On the day prior to an experiment, the animals are transferred to the experimental room. On the day of the experiment, various doses of test drugs or saline are injected intraperitoneally. After 60 min 2.5 mg/kg 6-chloro-2-(1-piperazinyl)pyrazine (MK-212) are injected intraperitoneally followed by decapitation after further 60 min. Trunk blood is collected and allowed to clot. Serum is obtained following centrifugation and stored at -20°C for the radioimmunoassay of corticosterone.

EVALUATION

Data are analyzed with a two-way analysis of variance. Differences between treatment groups are evaluated using the Student-Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Korte et al. (1991) studied the effect of a 5-HT_{1A} agonist on behavior and plasma corticosterone levels in

male Wistar rats before and after psychological stress of defeat.

Broqua et al. (1992) measured corticosterone and glucose levels in blood together with parameters of 5-HT metabolism in brain in stressed animals treated with the anti-depressant tianeptine.

Rittenhouse et al. (1992) measured plasma concentrations of renin, corticosterone, ACTH, and prolactin in rats after treatment with a 5-HT_{1A} agonist in three stress paradigms: immobilization, forced swim and conditioned fear.

Groenink et al. (1995) studied the corticosterone secretion in rats after application of 5-HT_{1A} receptor agonists and antagonists.

Aulakh et al. (1988, 1993) reported higher baseline levels of plasma corticosterone in fawn-hooded rats relative to Wistar and Sprague-Dawley strain rats. Long-term treatment (21 days) with antidepressant drugs significantly decreased plasma corticosterone in fawn-hooded rats. The authors recommended this strain as a genetic model of depression.

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E.2.7

Benzodiazepine Dependence

E.2.7.1

General Considerations

Benzodiazepine dependence is a hypothetical construct for the adaptive changes that occur as a result of chronic drug exposure. Two measures are usually considered to reflect dependence: the development to a drug's effects, and the abstinence signs of drug withdrawal.

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E.2.7.2

Benzodiazepine Tolerance and Dependence in Rats

PURPOSE AND RATIONALE

Induction of benzodiazepine tolerance and physical dependence has been reported for several animal species, such as rats, mice, dogs and monkeys. Ryan and Boisse (1983) and Boisse et al. (1986) developed a reproducible model exemplified for chlordiazepoxide in rats.

PROCEDURE

Male Sprague Dawley rats weighing 350–575 g are used. Chordiazepoxide hydrochloride is administered as solution 75 mg/ml by gavage after an initial loading dose of 450 mg/kg given at 7:00 A.M. and 5:00 P.M. Impairment of motor function is evaluated by a neurological screen including five different ladder and open field tests.

| Neurological Test | Maximum Depression Points |
|----------------------|---------------------------|
| Ladder, head down | 2 |
| Ladder, head up | 4 |
| Ladder, grasp reflex | 1 |
| Motor activity | 3 |
| Walking | 5 |
| Maximum | 15 |

The animals are rated by three independent observers. Once dependence is revealed, lower doses are used which are then increased in appropriate steps to induce an average depression rating of about five. Treatment is continued for 5 weeks during which time the dose

has to be increased. The degree of increase (about 5-fold for in the case of chlordiazepoxide) reflects the **tolerance** of the test compound.

To test **dependence**, rats are challenged with the benzodiazepine receptor antagonist Ro 15–1788 (flumazenil; ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5a)(1,4)benzodiazepine-3-carboxylate) at a dose of 25 mg/kg i.p. Withdrawal reactions are recorded just before, and 5, 15, 30 and 60 min after administration of the antagonist. Motor, autonomic and behavioral signs are monitored by operational defined criteria, such as position of the claws, salivation and diarrhea. The total withdrawal expression for each observation and each animal is estimated by summing the grades of all signs as recorded by three independent observers.

EVALUATION

For each animal and observation time, the scores are estimated by the average of all co-observers. From these estimates group means are computed and compared by *t*-test between control and test and by paired *t*-test for self-control comparisons before and after antagonist administration

MODIFICATIONS OF THE METHOD

Further studies in *rat* with benzodiazepines were performed by Vellucci and File (1979), Treit (1985) and Nath et al. (1997).

File (1985) found very different rates at which tolerance develops to the sedative, anticonvulsive and anxiolytic actions of benzodiazepines.

Bonnafous et al. (1995) studied the increase of gastric emptying induced by benzodiazepine withdrawal in rats. Gastric emptying was measured with a test meal containing ⁵¹Cr sodium chromate administered in rats, either previously receiving 15 mg/kg diazepam or DMSO i.p. for 7 days.

Benzodiazepine-like dependence potential of a putative 5-HT_{1A} agonist anxiolytic was assessed in rats by Goudie et al. (1994).

Studies in *mice* on tolerance and physical dependence with benzodiazepines were performed by Patel et al. (1988), Gallaher et al. (1986), Stephens and Schneider (1985), Nutt and Costello (1988) and Piot et al. (1990).

These phenomena were studied in *dogs* by McNicolas et al. (1988) and Löscher et al. (1989).

Studies in monkeys were performed by Lukas and Griffiths (1982), Yanagita (1983), Lamb and Griffiths (1984) and France and Gerak (1997).

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E.2.8

Genetically Modified Animals in Psychopharmacology

E.2.8.1

General Considerations

The use of genetically altered animals in biological research has also affected psychopharmacology. Genetically engineered strains of mice, modified by transgenesis or gene targeting (“knockout”) have been generated and are used as research tools for deciphering the genetic basis of behavior. These animals are designed to evaluate the efficacy of new pharmacological and gene therapy treatments in human hereditary diseases (Crnic 1996; Mayford et al. 1997; Costentin 1998; Picciotto and Wickmanm 1998; Anagnostopoulos et al. 2001).

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E.2.8.2

Special Reports on Genetically Altered Animals Useful for Evaluation of Drugs Against Anxiety

Wilson et al. (1996) reported that the **transgenic (mREN2) rat**, basically a model of genetically engineered hypertension, showed an anxiogenic profile in several tests, e. g., open field or elevated X-maze.

Saudou et al. (1994) found enhanced aggressive behavior in **mice lacking the 5-HT_{1B}receptor**. When confronted with an intruder, mutant mice attacked the intruder faster and more violently than did wild-type mice.

Trillat et al. (1998) studied the antidepressant effect of selective serotonin reuptake inhibitors (SSRIs) in homozygote mice deficient in the serotonin **5-HT_{1B}receptor**. The findings suggested that 5-HT_{1B} autoreceptors limit the effect of SSRIs particularly in the hippocampus while postsynaptic 5-HT_{1B} receptors are required for the antidepressant activity of SSRIs.

Parks et al. (1998) described increased anxiety in **mice lacking the serotonin 1A receptor**. Mice with an inactivated gene encoding the 5-HT_{1A} receptor have an increased tendency to avoid a novel and fearful environment and to escape a stressful situation.

Likewise, Heisler et al. (1998) found elevated anxiety and antidepressant-like responses in serotonin **5-HT_{1A} mutant mice**.

Ramboz et al. (1999) described **serotonin receptor 1A knockout mice** as an animal model of anxiety-related disorder. The authors demonstrated that mice without 5-HT_{1A} receptors display decreased exploratory activity and increased fear of aversive environments (open or elevated spaces). 5-HT_{1A} knockout mice also exhibited a decreased immobility in the forced swim test, an effect commonly associated with antidepressant treatment. These results showed that 5-HT_{1A} receptors are involved in the modulation of exploratory and fear-related behaviors and suggested that reductions in 5-HT_{1A} receptor density due to genetic defects or environmental stressors might result in heightened anxiety.

Gross et al. (2000) used genetically altered mice to determine the influence of different neurotransmitter receptors on fear and anxiety. **Mice with a genetic deletion of the 5-HT_{1A} receptor** were more fearful in a number of behavioral conflict tests, confirming the importance of this receptor in modulating anxiety.

Knapp et al. (2000) bred rats selectively for high or low hypothermic responses to the specific 5-HT_{1A} receptor agonist 8-hydroxy-2-di-n-propylamino tetralin (8-OH-DPAT). These rats differed in responses related to anxiety and depression.

Holmes (2001) reviewed targeted gene mutation approaches to the study of anxiety-like behavior in mice.

Schramm et al. (2001) found that the α_{2A} -adrenergic receptor plays a protective role in mice behavioral models of depression and anxiety. The genetic **knockout of the α_{2A} -adrenergic receptor** makes mice less active in a modified Porsolt's swim test and insensitive to the antidepressant effect of imipramine in this paradigm. Furthermore, α_{2A} -adrenergic receptor knockout mice appear more anxious than wild-type C57 Bl/6 mice in rearing and light/dark models of anxiety.

Quinlan et al. (2000) reported that **mice lacking the long splice variant of the gamma 2 subunit of the GABA_A receptor** are more sensitive to benzodiazepines. Lack of the gamma 2L subunits may shift the GABA_A receptor from an inverse-agonist-prefering toward an agonist-prefering configuration.

Yamada et al. (2000) described neurobehavioral alterations in mice with a targeted **deletion of the tumor necrosis factor-alpha gene** and the implications for emotional behavior.

Miyakawa et al. (2001) studied the behavior of **mice lacking the M₁ muscarinic acetylcholine receptor**. The animals exhibited a pronounced increase in locomotor activity in various tests, including open field, elevated plus maze, and light/dark transition tests. However, hippocampus-dependent learning was intact.

Piccioletto et al. (2001) studied physiological and behavioral phenotypes of **neuronal nicotine acetylcholine receptor subunit knockout mice** and discussed possible implications for drug development.

Rupniak et al. (2001) used assays for antidepressant and anxiolytic drugs, such as resident-intruder or the forced swim test, to compare the phenotype of **NK1R^{-/-} mice** with pharmacological blockade of the substance P (NK1) receptor.

Montag-Sallaz and Montag (2003) found severe cognitive and motor coordination deficits in **Tenascin-R-deficient mice**. The animals were tested for grip strength, open field behavior, elevated plus maze, light-dark avoidance, hole board examination, in the Morris water maze and in two-way active avoidance learning using several systems from TSE Systems, Bad Homburg, Germany).

Bilkei-Gorzo et al. (2004) studied behavioral and drug effects in **pre-proenkephalin-deficient mice** in the elevated zero-maze maze (using the VideoMot 2 system), the light-dark test using the animal activity monitor Actimot, and in the startle response test (all systems provided by TSE Systems, Bad Homburg, Germany)

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- nal, electrical or chemical stimuli to various areas of the brain. After 10 to 15 days of once-daily stimulation, the duration and intensity of after-discharges reach a stable maximum and a characteristic seizure is produced. Subsequent stimulation then regularly elicits seizures.
- Surveys of methods being used to test compounds with anticonvulsant properties have been provided by Toman and Everett (1964), Woodbury (1972), Hout et al. (1973), Swinyard (1973), Koella (1985), Meldrum (1986), Rump and Kowalczyk 1987; Löscher and Schmidt (1988), Fisher (1989), Rogawski and Porter (1990), Porter and Rogawski (1992).
- Epilepsy becomes drug resistant in 20%–30% of patients. Out of the animal models, the amygdala-kindled rat seems to be a suitable approach (Löscher 1997, 1998, 2002a, 2002b). Furthermore, the rat cortical dysplasia model is recommended (Smyth et al. 2002).
- Several biochemical hypotheses have been advanced, involving the inhibitory GABAergic system and the system of the excitatory amino acids glutamate and aspartate. Excitatory receptors have been divided into subtypes according to the actions of specific agonists or antagonists. Agents which reduce GABA_A synaptic function provoke convulsions. A convulsive state is induced by the direct blockade of GABA_A receptors (e. g. to the action of bicuculline) or a reduction in the GABA-mediated opening of the chloride ion channel (e. g., by picrotoxin). One major factor in epileptogenesis seems to be a decreased function of GABA_A synapses.
- More recently, research has focused on the therapeutic potential of blocking excitatory amino acids – in particular glutamate. Of the three receptors of glutamate, the NMDA (N-methyl-D-aspartate)-receptor is considered the one of most interest in epilepsy and competitive NMDA receptor antagonists are proposed as potential anti-epileptic drugs. Excessive excitatory amino acid neurotransmission is thought to be associated with the neuropathologies of epilepsy, stroke and other neurodegenerative disorders. Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987).
- Fabene and Sbarbati (2004) underlined the value of *in vivo* MRI in different models of experimental epilepsy.

E.3

Anti-Epileptic Activity

E.3.0.1

General Considerations

Epilepsy is a disease of high prevalence, being well known since thousands of years as “morbus sacer”. In spite of intensive investigations, the pathophysiology of epilepsy is still poorly understood. Studies with various animals models have provided ample evidence for heterogeneity in the mechanisms of epileptogenesis. New evidence derives from investigations of kindling, which involves the delivery of brief, initially sublimi-

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E.3.1**In Vitro Methods****E.3.1.1****³H-GABA Receptor Binding**

See Sect. E.2.1.1.

E.3.1.2**GABA_A Receptor Binding**

See Sect. E.2.1.2.

E.3.1.3**GABA_B Receptor Binding**

See Sect. E.2.1.3.

The *in vitro* assays for GABA-ergic compounds, described in the Sect. E.2 (anxiolytics) are similarly used for evaluation of anti-epileptic compounds.

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E.3.1.4**³H-GABA Uptake in Rat Cerebral Cortex Synaptosomes****PURPOSE AND RATIONALE**

Roberts (1974) and others have proposed that the inhibitory action of the amino acid γ -aminobutyric acid (GABA) is the fine tuning control for pacemaker neurons. Disruption of this interplay due to inadequacies of the GABA system result in various disorders, in particular convulsive seizures (Roberts 1974; Korgsgaard-Larsen 1985). The nonspecific action of GABA-mimetics makes inhibition of the uptake mechanism, which terminates the neurotransmitters action, the ideal choice for increasing GABA's concentration at specific sites (Roberts 1974; Tapia 1975; Meldrum et al. 1982; Brehm et al. 1979). Demonstration of the high affinity mechanism that best reflects the *in vivo* condition utilizes GABA depleted cerebral cortex synaptosomes (Ryan and Roskoski 1977; Iversen and Bloom 1972; Roskoski 1978).

Although the physiological role of GABA transport systems is still unclear, uptake inhibitors such as THPO [4,5,6,7-tetrahydroisoxazolo-(4,5-C)pyrid-3-ol], nipecotic acid, cis-4-hydroxynipecotic acid, and guvacine exhibit anticonvulsant effects (Meldrum et al. 1982; Brehm et al. 1979). Furthermore, a number of neuroleptics have been shown to inhibit GABA uptake (Fjalland 1978). In particular, fluspirilene was found to be equivalent to the most potent uptake inhibitors known.

The assay is used as a biochemical screen for potential anticonvulsants or GABA (γ -aminobutyric acid) mimetic compounds that act by inhibiting GABA uptake.

PROCEDURE

Reagents

- 0.5 M Tris buffer, pH 7.4
- Ringer's solution+10 mM Tris buffer, pH 7.4 containing
 - glucose 10.0 mM,
 - NaCl 150.0 mM,
 - KCl 1.0 mM,
 - MgSO₄ 1.2 mM,
 - Na₂HPO₄ 1.2 mM.
- Depolarizing Ringer's solution, pH 7.4 reagent 2 containing
 - KCl 56 mM,
 - CaCl₂ 1 mM.
- 0.32 M sucrose
- ³H-GABA is diluted to 2.5×10^{-4} M with distilled water. Forty μ l of this solution in 1 ml of reaction mixture will yield a final concentration of 10^{-5} M.
- Test compounds

A 10 mM stock solution is made up in distilled water, ethanol, or DMSO and serially diluted, such that the final concentration in the assay ranges from 10^{-3} to 10^{-8} M. Total and nonspecific controls should use solvent of test compound.

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Cerebral cortex is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem homogenizer. The homogenate is centrifuged at 1000 g for 10 min. The supernatant (S₁) is decanted and recentrifuged at 1000 g for 10 min. The pellet (P₂) is resuspended in 9 volumes of 0.32 M sucrose and centrifuged at 24,000 g for 10 min. The

washed pellet is resuspended in 15 volumes of depolarizing Ringer's solution, incubated at 25°C for 10 min and centrifuged at 3000 g for 10 min. The resulting pellet is resuspended in 15 volumes of Ringer's solution and is ready for use.

Assay

- 60 μ l Ringer's solution,
- 100 μ l vehicle or appropriate drug concentration,
- 800 μ l tissue suspension.

Microcentrifuge tubes are set up in triplicate. Nonspecific controls are incubated at 0°C and totals at 25°C for 10 min. Forty μ l of ³H-GABA are added and the tubes are reincubated for 10 min. All tubes are centrifuged at 13,000 g for 1 min. The supernatant is aspirated and 1 ml of solubilizer (Triton X-100 + 50% EtOH, 1:4, v/v) is added and mixed to dissolve pellets. Tubes are incubated at 90°C for 3 min, then centrifuged at 13,000 g for 15 min. 40 μ l of supernatant is counted in 10 ml Liquiscint scintillation cocktail.

EVALUATION

Active uptake is the difference between cpm at 25°C and 0°C. The percent inhibition at each drug concentration is the mean of three determinations. IC₅₀ values are derived from log-probit analysis.

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E.3.1.5

GABA Uptake and Release in Rat Hippocampal Slices

PURPOSE AND RATIONALE

The GABA transporter, the subsynaptic GABA_A-receptor, and the GABA_B-autoreceptor are therapeutically the most relevant targets for drug actions influencing GABAergic synaptic transmission. Uptake inhibitors are potential anticonvulsants.

PROCEDURE

For measurement of GABA uptake, rat hippocampal slices are cut with a McIlwain tissue slicer (100 μ m-thick prisms) and dispersed in ice-cold Krebs-Ringer solution with HEPES buffer (pH 7.4). Following two washes, slices (15 mg) are incubated at 37°C for 15 min in the presence or absence of test compound. [³H]-GABA is added and samples are incubated for an additional 5 min before filtration through Whatman GF/F filters. Samples are then washed twice with 5 ml ice-chilled 0.9% saline. Distilled water is added and samples are allowed to sit at least 60 min before measured for radioactivity by liquid scintillation spectroscopy. Blanks are treated in an identical manner but are left on ice throughout the incubation.

For measurement of GABA release, rat hippocampal slices are prepared and dispersed in ice-cold HEPES-buffered (pH 7.2) Krebs Ringer solution and incubated with 0.05 μ M [³H]-GABA for 15 min at 37°C. Following two washes, the slices are incubated for an additional 15 min and finally resuspended in medium. Tissue (10 mg) is incubated at 37°C for a 15 min release period in the presence or absence of test compound. At the end of the release period, the medium is separated from tissue by centrifugation at 500 g for approximately 1 min and poured into 0.5 ml of perchloric acid (0.4 N). The tissue is homogenized in 0.13 N perchloric acid. Radioactivity in the samples is measured by using liquid scintillation spectroscopy.

EVALUATION

For GABA-uptake, *IC*₅₀-values (μ M) are determined.

In GABA-release experiments, results are expressed as the amount of radioactivity released as a percent of the total radioactivity.

MODIFICATIONS OF THE METHOD

Roskoski (1978) studied the net uptake of GABA by high affinity synaptosomal transport systems.

Nilsson et al. (1990, 1992) tested GABA uptake in astroglial primary cultures.

The **isolated nerve-bouton preparation** was used to study GABA release (Jang et al. 2001; Kishimoto et al. 2001; Akaike et al. 2002; Akaike and Moorhouse 2003). The technique was developed by Drewe et al. (1988), Vorobjev (1991), Haage et al. (1998), Rhee et al. (1999), and Koyama et al. (1999).

The method is based on the local application of mechanical vibration directly to the chosen site of a brain slice and does not require the enzymatic pretreatment of the tissue. The mechanical vibration is applied via a glass rod (0.5 mm in diameter) mounted on a piezoelectric bimorph crystal at the site of the chosen brain tissue. The dissociated cells are allowed to settle at the bottom of a Petri dish for 20 min. The cell bodies are usually 10–15 μ m at their longest axis, rounded or elongated in shape. Some cells had remaining neurites up to 100 μ m long. The majority of cells had neurites less than 15 μ m long.

In other studies (Koyama et al. 1999; Kishimoto et al. 2001), a custom-built vibrating stylus was placed in the appropriate region for mechanical dissociation. The glass capillary (1.5 mm o.d.) was pulled to a fine tip and fire-polished. The tip was placed within the appropriate region by a manipulator. The vibrating stylus was driven by an electronic relay and the tip was horizontally moved (excursions of 2–3 mm at 0.5–2 Hz) for 2 min.

Neurons with adherent functional synaptic terminals were investigated by tight-seal whole-cell recordings from the postsynaptic cells.

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- that were originally named after reasonably selective ligands: *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate (Cotman and Iversen 1987; Watkins and Olverman 1987; Collingridge and Lester 1989; Monaghan et al. 1989; Carlsson and Carlsson 1990; Young and Fagg 1990; Nakanishi 1992; Cunningham et al. 1994; Herrling 1994; Iversen and Kemp 1994; Mayer et al. 1994; Meldrum and Chapman 1994; Monaghan and Buller 1994; Watkins 1994; Bettler and Mülle 1995; Fletcher and Lodge 1995; Becker et al. 1998; Danysz and Parsons 1998; Meldrum 1998; Chittajallu et al. 1999; Dingledine et al. 1999; Hatt 1999; Gallo and Ghiani 2000; Lees 2000; Meldrum 2000). It turned out that NMDA, AMPA and kainate receptor subunits are encoded by at least six gene families as defined by sequence homology: a single family of AMPA receptors, two for kainate, and three for NMDA (Dingledine et al. 1999; Mayer and Armstrong 2004).
- The NMDA subtype is a hetero-oligomer consisting of an NR1 subunit combined with one or more NR2 subunits and a third subunit, NR3 (Loftis and Janowsky 2003). The receptor has two amino acid recognition sites, one for glutamate and one for glycine, both of which must be occupied to promote channel opening. A variety of drugs have been identified which block the channel selectively (Bräuner-Osborne et al. 2000; Kemp and McKernan 2002).
- The AMPA subtype is a hetero-oligomer formed from combinations of iGluR1–4. Selective agonists and competitive antagonists acting at the glutamate recognition site have been useful for defining the physiological and pathophysiological roles played by the receptor. AMPA receptor modulators have been discussed as cognitive enhancers (Lynch 2004).
- The kainate subtype consists of hetero-oligomers, comprising five subunits (Hollmann and Heinemann 1994; Huettner 2003).
- Excessive excitatory amino acid neurotransmission has been associated with the neuropathologies of epilepsy, stroke and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and Olverman 1987; Parsons et al. 1998). Antagonism of NMDA receptor function appears to be the mechanism of action of some anticonvulsant and neuroprotective agents (Loscher 1998; Tauboll and Gjerstad 1998). The binding site for [³H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized (Thomsen 1997). NMDA receptors are believed to be coupled to a cation channel

E.3.1.6

Glutamate Receptors: [³H]CPP Binding

PURPOSE AND RATIONALE

The ionotropic glutamate receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain. The family comprises three pharmacologically defined classes

which converts to an open state with NMDA receptor activation (Kemp et al. 1987; Mukhin et al. 1997). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+} and Zn^{2+} . Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and novel anticonvulsants, such as MK-801, block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as CPP and the phosphono analogs of L-glutamate, AP7 and AP5 (2-amino-5-phosphonopentanoic acid), are inhibitors at the excitatory amino acid binding site (Olverman et al. 1986; Davies et al. 1986; Harris et al. 1986; Murphy et al. 1987; Lehmann et al. 1987).

The following assay is used to assess the affinity of compounds for the excitatory amino acid binding site of the NMDA receptor complex. [3H]CPP 3-[(\pm)-2-carboxypiperazin-4-yl]-1-phosphonic acid is a structurally rigid analog of the selective NMDA receptor antagonist 2-AP7 (2-amino-7-phosphonoheptanoic acid).

PROCEDURE

Reagents

1. Buffer A: 0.5 M Tris HCl, pH 7.6
60.0 g Tris HCl
13.9 g Tris Base
q.s. to 1 liter with distilled water
2. Buffer B: 50 mM Tris HCl, pH 7.6
Dilute buffer A 1:10 with distilled water
3. L-Glutamic acid, 5×10^{-3} M
Dissolve 7.36 mg of l-glutamic acid (Sigma G1251) with 10.0 ml distilled water. Aliquots of 20 μ l to the assay tube will give a final concentration of 10^4 M.
4. [3H]CPP is obtained from New England Nuclear, specific activity 25–30 Ci/mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 μ l are added to each tube to yield a final concentration of 10 mM.
5. Test compounds. A stock solution of mM is made with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.
6. Triton-X 100, 10% (v/v) (National Diagnostics, EC-606) A stock solution of Triton-X100, 10%, can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton-X100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1:15 dilution) is preincubated with an aliquot of Triton-X 100, 10%, to give a final concentration of 0.05% (v/v).

Tissue Preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1000 g for 10 min. (SS34, 3000 rpm, 4°C). The supernatant is centrifuged at 20,000 g (SS34, 12,000 rpm, 4°C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 60, 15 s) and spin at 7600 g (SS34, 8000 rpm, 4°C) for 20 min. Save the supernatant, swirl off the upper buffy layer of the pellet and add to the supernatant. Centrifuge the supernatant at 48,000 g (SS34, 20,000 rpm, 4°C) for 20 min. Resuspend the pellet with 15 volumes of cold distilled water and centrifuge. Discard the supernatant and store the pellet at -70°C .

On the day of the assay, resuspend the pellet in 15 volumes ice-cold 50 mM Tris buffer, pH 7.6. Preincubate the homogenate with Triton-X in a final concentration 0.05% (v/v) for 15 min at 37°C with agitation. Centrifuge the homogenate at 48,000 g (SS34, 20,000 rpm, 4°C for 20 min. Wash the pellet an additional 3 times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 20 times the original wet weight.

Assay

1. Prepare assay tubes in triplicate.

| | |
|-------------|---------------------------------------------------------------------------------------------|
| 380 μ l | distilled water |
| 50 μ l | buffer A, 0.5 M Tris HCl, pH 7.6 |
| 20 μ l | L-glutamic acid, 10^{-4} M, or distilled water, or appropriate concentration of inhibitor |
| 50 μ l | [3H]CPP |
| 500 μ l | tissue homogenate |
2. Following the addition of the tissue, the tubes are incubated for 20 min at 25°C with agitation. Place the tubes in an ice bath at the end of the incubation. Terminate the binding by centrifugation (HS4, 7000 rpm, 4°C) for 15 min. Return the tubes to ice. Aspirate and discard the supernatant. Carefully rinse the pellet three times with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml and add an additional 4 ml scintillation fluid.

EVALUATION

Specific binding is determined from the difference of binding in the absence of presence of 10^{-4} M L-

glutamic acid and is typically 60–70% of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

MODIFICATIONS OF THE ASSAY

Glutamate (Non Selective)

The assay measures the binding of glutamate, which binds non selectively to ionotropic glutamate receptors including the NMDA, AMPA, and kainate subtypes (Foster and Fagg 1987). In addition, glutamate binds to a family of metabotropic glutamate receptors.

Whole brains (except cerebellum) are obtained from male Wistar rats. A membrane fraction is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1.6 nM [3 H]L-glutamate for 10 min at 37°C. Non-specific binding is estimated in the presence of 50 μ M L-glutamate. Membranes are filtered and washed 3 times to separate bound from free ligand and filters are counted to determine [3 H]L-glutamate bound.

Convulsions induced in mice by intravenous injections of 2.0 mmol/kg L-glutamic acid can be inhibited by glutamate antagonists (Piotrovsky et al. 1991).

Glutamate AMPA

The assay measures the binding of [3 H]AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), a selective agonist which binds to the AMPA receptor subtype of glutamate-gated ion channels (Honore et al. 1982; Olsen et al. 1987; Fletcher and Lodge 1995).

Membranes are prepared from male rat brain cortices by standard techniques. Fifteen mg of membrane preparation is incubated with 5 nM [3 H]AMPA for 90 min at 4°C. Non-specific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed 3 times and the filters are counted to determine [3 H]AMP bound.

Mutel et al. (1998) recommended [3 H]Ro 48–8587 as specific for the AMPA receptor.

Fleck et al. (1996) described AMPA receptor heterogeneity in rat hippocampal neurons. AMPA receptor antagonists were described by Kohara et al. (1998), Wahl et al. (1998), Kodama et al. (1999), Nielsen et al. (1999) and reviewed by Chimirri et al. (1999).

Glutamate Kainate

The assay measures the binding of [3 H]kainate, a selective agonist that binds to the kainate subtype of the ionotropic glutamate receptors in rat brain (London and Coyle 1979; Clarke et al. 1997).

Whole brains (except cerebellum) are obtained from male Wistar rats. Fifteen mg of a membrane fraction prepared by standard techniques is incubated with 5.0 nM [3 H]kainate for 1 h at 4°C. Non-specific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed 3 times to separate free from bound ligand and filters are counted to determine [3 H]kainate bound.

Toms et al. (1997), Zhou et al. (1997) recommended [3 H]-(2S,4R)-4-methylglutamate as kainate-receptor selective ligand.

Irreversible inhibition of high affinity [3 H]kainate binding by a photoactivatable analogue was reported by Willis et al. (1997).

Worms et al. (1981) described the behavioral effects of systemically administered kainic acid.

Hu et al. (1998) described neuronal stress and seizure-induced injury in C57/BL mice after systemic kainate administration.

Glutamate NMDA-Agonist Site

The assay measures the binding of CGS 19755, a selective antagonist, to the agonist site of the NMDA receptor (Lehmann et al. 1988; Murphy et al. 1988; Jones et al. 1989)

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E.3.1.7

NMDA Receptor Complex: [³H]TCP Binding

PURPOSE AND RATIONALE

The purpose of this assay is to determine the binding affinity of potential noncompetitive NMDA antagonists at the phencyclidine (PCP) binding site which is believed to be within or near the NMDA-regulated ion channel. TCP, 1-[1-(2-thienyl)cyclohexyl]-piperidine, is a thienyl derivative of PCP.

Excessive activity of excitatory amino acid neurotransmitters has been associated with the neuropathologies of epilepsy, stroke and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and Olverman 1987). Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA) and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). The binding site for [³H]2-

amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized. NMDA receptors are believed to be coupled to a cation channel which converts to an open state following activation (Kemp et al. 1987). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+} , Zn^{2+} and polyamines (Loo et al. 1986; Snell et al. 1987; Reynolds et al. 1988; Thomson 1989; Snell et al. 1988; Sacaan and Johnson 1989; Thedinga et al. 1989; Williams et al. 1989). Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and the neuroprotective agent MK-801 block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid (CPP), and the phosphono analogs of L-glutamate, 2-amino-7-phosphonoheptanoic acid (2-AP7) and 2-amino-5-phosphonopentanoic acid (2-AP5), are inhibitors at the excitatory amino acid recognition site.

Molecular cloning and functional expression of rat and mouse NMDA receptors (Moriyoshi et al. 1991; Meguro et al. 1992), a family of AMPA-selective glutamate receptors (Keinänen et al. 1990) and the metabotropic glutamate receptors mGluR1–mGluR6 (Schoepp et al. 1990; Masu et al. 1991; Abe et al. 1992; Bashir et al. 1993; Nakajima et al. 1993; Tanabe et al. 1993) have been reported.

PROCEDURE I

Reagents

1. Buffer A: 0.1 M HEPES, pH 7.5
Weigh 23.83 g HEPES
Add approximately 900 ml distilled water.
Adjust pH to 7.5 with 10 N NaOH.
q.s. to 1 liter with distilled water.
2. Buffer B: 10 mM HEPES, pH 7.5
Dilute buffer A 1:10 with distilled water and adjust pH to 7.5.
3. L-Glutamic acid, 5×10^{-3} M
Dissolve 7.36 mg with 10.0 ml distilled water.
Aliquots of 20 μ l to the assay tube will give a final concentration of 10^{-4} M.
4. Glycine, 5×10^{-4} M
Dissolve 3.75 mg with 10.0 ml distilled water.
Dilute 1:10 with distilled water.
Aliquots of 20 μ l to the assay tube will give a final concentration of 10^{-5} M.
5. Phencyclidine HCl (PCP) is used for nonspecific binding.
Dissolve 0.7 mg in 0.5 ml distilled water.

Aliquots of 20 μ l to the assay tube will give a final concentration of 10^{-4} M.

6. [3 H]TCP is obtained from New England Nuclear, specific activity 42–60 Ci/mmol. For IC_{50} determinations, a 50 nM stock solution is made with distilled water. Aliquots of 50 μ l are added to each tube to yield a final concentration of 2.5 nM.
7. Test compounds. A stock solution of 5 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Cerebral cortex of male Wistar rats, 7–10 weeks of age, is dissected over ice and homogenized in ice-cold 0.32 M sucrose, 30 volumes of original tissue weight, for 60 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1000 g for 10 min. (SS34, 3000 rpm, 4°C). The supernatant is centrifuged at 20,000 g for 20 min. (SS34, 12,000 rpm, 4°C). The pellet is resuspended with cold distilled water, to 50 volumes of original tissue weight, using the Tissumizer, 60 s at setting of 70. The homogenate is incubated at 37°C for 30 min, transferred to centrifuge tubes, and centrifuged at 36,000 g for 20 min. (SS34, 16,500 rpm, 4°C). The pellet is again resuspended in 50 volumes distilled water, incubated and centrifuged. All resuspensions with the Tissumizer are for 60 s at a setting of 70. The resulting pellet is resuspended in 30 volumes of ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged, and washed once again (resuspension and centrifugation) with buffer. Following resuspension in 30 volumes of buffer, the homogenate is frozen in the centrifuge tube and stored at -70°C until the day of the assay.

On the day of the assay, the homogenate is thawed and centrifuged at 36,000 g for 20 min. (SS34, 16,500 rpm, 4°C). The pellet is washed three times by resuspension with ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged and finally resuspended in 30 volumes of buffer. Aliquots of 500 μ l are used for each assay tube, final volume 1000 μ l, and correspond to approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate. For each test compound, inhibition of [3 H]TCP binding is measured both in the absence (basal) and presence (stimulated) of 100 μ M L-glutamic acid and 10 mM glycine.

| Basal | Stimulated | |
|-------------|-------------|--------------------------------------------------------------------------------|
| 380 μ l | 340 μ l | Distilled water |
| 50 μ l | 50 μ l | Buffer A, 0.1 M HEPES, pH 7.5 |
| 20 μ l | 20 μ l | PCP (reagent A5) or distilled water, or appropriate concentration of inhibitor |
| 0 μ l | 20 μ l | L-glutamic acid (reagent A3) |
| 0 μ l | 20 μ l | Glycine (reagent A4) |
| 50 μ l | 50 μ l | [³ H]TCP (reagent A6) |
| 500 μ l | 500 μ l | Tissue homogenate |

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25°C with agitation. The assay is terminated by separating the bound from non-bound radioligand by rapid filtration with reduced pressure over Whatman GF/B filters, pre-soaked in 0.05% polyethylene-imine, using the Brandell cell harvesters. The filters are rinsed once with buffer before filtering the tubes, and rinsed two times after filtration. The filters are counted with 10 ml Liquiscint.

EVALUATION

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M PCP. Specific binding is typically 50% of total binding in basal conditions, and 90% of total binding when stimulated by L-glutamic acid and glycine. L-Glutamic acid and glycine typically increase specific binding to 300% and 200% of basal binding, respectively. The combination of L-glutamic acid and glycine typically produce a greater than additive effect, increasing specific binding to 700% of basal binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

Protocol Modification for Crude Membrane Homogenates

This modified procedure for the preparation of membrane homogenates does not use extensive lysing and washing of the tissue to remove endogenous L-glutamate, glycine and other endogenous compounds which enhance [³H]TCP binding. This procedure may be used for rapid screening of compounds for inhibition of [³H]TCP binding site without specifically defining an interaction at the ion channel or modulatory sites of the NMDA receptor complex.

PROCEDURE II

Reagents

1. Buffers A and B are prepared as described above.
2. Phencyclidine HCl is used for nonspecific binding and is prepared as described above.

3. [³H]TCP is prepared as described above.
4. Test compounds are prepared as described above.

Tissue Preparation

Cortical tissue is dissected, homogenized in 30 volumes of 0.32 M sucrose and a crude P₂ pellet is prepared as described above. The pellet is resuspended in 30 volumes of 10 mM HEPES, pH 7.5, centrifuged at 36,000 g (SS34, 16,500 rpm, 4°C) for 20 min, and again resuspended in 100 volumes of buffer. This homogenate is used directly in the assay in aliquots of 500 μ l.

Assay

1. Prepare assay tubes in triplicate.

| Volume | Solution |
|-------------|------------------------------------------------------------------------------|
| 380 μ l | Distilled water |
| 50 μ l | Buffer A, 0.1 M HEPES, pH 7.5 |
| 20 μ l | PCP (reagent IA5) or distilled water, appropriate concentration of inhibitor |
| 50 μ l | [³ H]TCP (reagent IA6) |
| 500 μ l | Tissue homogenate |

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25°C with agitation. The assay is terminated by rapid filtration as described above. The filters are rinsed and counted for bound radioactivity as above.

EVALUATION

Specific binding is determined from the difference of binding in the presence or absence of 10^{-4} M PCP. Specific binding is typically 90% of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis.

MODIFICATIONS OF THE METHOD

Instead of [³H]TCP, radiolabeled [³H]MK-801 has been used as ligand (Wong et al. 1988; Javitt and Zukin 1989; Williams et al. 1989).

Sills et al. (1991) described [³H]CGP 39653 as a N-methyl-D-aspartate antagonist radioligand with low nanomolar affinity in rat brain.

Nowak et al. (1995) reported that swim stress increases the potency of glycine to displace 5,7-[³H]dichlorokynurenic acid from the strychnine-insensitive glycine recognition site of the N-methyl-D-aspartate receptor complex.

NMDA receptor cloning studies have shown that NMDA receptors contain at least one of seven different NMDAR1 subunits (NR1A–NR1G) (Sugihara

et al. 1992) and at least one of four NMDAR2 subunits (NR2A–NR2D) (Kutsuwada et al. 1992; Ishii et al. 1993). While the NR1 subunits are generated by alternative splicing of a single gene, the NR2 subunits are products of four highly homologous genes. Thus, there are thousands of potential subunit combinations yielding complexes of four or five subunits.

Grimwood et al. (1996) reported generation and expression of stable cell lines expressing recombinant human NMDA receptor subtypes, two cell lines expressing NR1a/NR2A receptors and one cell line expressing NR1a/NR2B receptors.

NR2B selective NMDA antagonists were described by Fischer et al. (1997), Kew et al. (1998), Reyes et al. (1998), Chenard and Menniti (1999).

For discovery of novel NMDA receptor antagonists, Bednar et al. (2004) developed a high-throughput functional assay based on fluorescence detection of intracellular calcium concentrations. Mouse fibroblasts L(tk-) cells expressing human NR1a/NR2B NMDA receptors were plated in 96-well plates and loaded with fluorescence calcium indicator fluo-3 AM. NR2B antagonists were added after stimulation of NMDA receptors with 10 μ M glutamate and 10 μ M glycine. Changes in fluorescence after addition of the antagonists were fitted with a single exponential equation providing k_{obs} .

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E.3.1.8

Metabotropic Glutamate Receptors

PURPOSE AND RATIONALE

In addition to ionotropic (AMPA, kainate and NMDA) receptors, glutamate interacts with a second family of receptors, metabotropic or mGlu receptors (Tanabe et al. 1992, 1993; Schoepp and Conn 1993; Hollmann and Heinemann 1994; Nakanishi and Masu 1994; Okamoto et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995, 1996; Pin and Duvoisin 1995; Conn and Pin 1997; Alexander et al. 2001; Skerry and Genover 2001; DeBlasi et al. 2001; Pin and Acher 2002; Conn 2003). Three groups of native receptors are distinguishable on the basis of similarities of agonist pharmacology, primary sequence, and G protein-effector coupling: Group I (mglu₁ and mglu₅ and splice variants) are coupled via G_{q/11} to phosphoinositide hydrolysis. Group II (mglu₂ and mglu₃) are negatively coupled via G_i/G_o to adenylyl cyclase and inhibit the formation of cAMP following exposure of cells to forskolin or activation of an intrinsic G_s-coupled receptor (e.g. adenosine A₂ receptor). The group III receptors (mglu₄, mglu₆, mglu₇, and mglu₈) also inhibit forskolin-stimulated adenylyl cyclase.

Various agonists and antagonists for metabotropic glutamate receptors were described (Ishida et al. 1990, 1994; Porter et al. 1992; Jane et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995; Annoura et al. 1996; Bedingfield et al. 1996; Thomsen et al. 1996; Acher et al. 1997; Doherty et al. 1997; Brauner-Osborne et al. 1998; Kingston et al. 1998; Monn et al. 1999; Jane and Doherty 2000). Schoepp et al.

(1999) reviewed pharmacological agents acting at subtypes of metabotropic glutamate receptors. Gssparini et al. (2002) described allosteric modulators of group I metabotropic glutamate receptors as novel subtype-selective ligands and their therapeutic perspectives.

Several radioligands for metabotropic glutamate receptors were described:

- for subtype mGluR4a receptor by Eriksen and Thomsen (1995),
- for group II mGlu receptors by Cartmell et al. (1998), by Ornstein et al. (1998), and by Schaffhauser et al. (1998).

Riedel and Reymann (1996) discussed the role of metabotropic glutamate receptors in hippocampal long-term potentiation and long-term depression and their importance for learning and memory. Furthermore, possible roles in the treatment of neurodegenerative disorders (Nicoletti et al. 1996; Bruno et al. 1998), and of Parkinson's disease (Konieczny et al. 1998) were discussed. Anticonvulsive properties (Attwell et al. 1998; Thompson and Dalby 1998; Gasparini et al. 1999) as well as anxiolytic properties (Helton et al. 1998) of metabotropic glutamate receptor ligands were reported. Christoffersen et al. (1999) found a positive effect on short-term memory and a negative effect on long-term memory of the class I metabotropic glutamate receptor antagonist, AIDA, in rats.

PROCEDURE

Cultured cells are prepared from cerebral cortex of 17-day embryos of Wistar rats. Prior to the experiments, the culture is maintained for 8–12 days with minimum essential medium (MEM) containing 5% fetal calf serum and 5% horse serum.

For **cyclic AMP assays**, the cultured cells are preincubated with HEPES-buffered Krebs-Ringer solution containing 5.5 mM glucose (HKR) for 1–1.5 h, then exposed to various agonists for 15 min in the absence or presence of 10 μ M forskolin. The content of cyclic AMP is measured using a radioimmuno-assay kit after homogenization with 0.1 M HCl.

For **phosphoinositide turnover assays**, the cultured cells are prelabeled with myo-1,2- $[^3\text{H}]$ inositol in MEM for 8–10 h. The cells are washed twice with HKR containing 10 mM LiCl, and then exposed to various agonists in HKR containing 10 mM LiCl for 30 min. The reaction is terminated with 2% trichloroacetic acid, and the homogenized samples are analyzed for inositol constituents by anion exchange chromatography (Berridge et al. 1982). The

extracts are applied to columns containing 1 ml of Dowex 1 in the formate form. The phosphate esters are then eluted by the step-wise addition of solutions containing increasing concentrations of formate. Glycero-phosphoinositol and inositol 1:2-cyclic phosphate are eluted with 5 mM-sodium tetraborate plus 150 mM sodium formate. The penultimate solution contains 0.1 M-formic acid plus 0.3 M-ammonium formate, followed by 0.1 M-formic acid plus 0.75 M-ammonium formate, each of which removes more polar inositol phosphates. The 1 ml fractions eluted from the columns are counted for radioactivity after addition of 10 ml of Biofluor.

The percentage of radioactivity of inositol phosphates to the total applied to the column is calculated.

EVALUATION

Dose-response curves for inhibition of forskolin-stimulated cAMP formation and for percentage of phosphoinositide hydrolysis are established for each test compound.

MODIFICATIONS OF THE METHOD

Thomsen et al. (1993, 1994) used baby hamster kidney (BHK) cells stably expressing mGluR_{1 α} , mGluR₂ or mGluR₄ for measurements of phosphoinositol hydrolysis or cAMP formation.

Varney and Suto (2000) recommended functional high throughput screening assay for the discovery of subtype-selective metabotropic glutamate receptor ligands.

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E.3.1.9

Excitatory Amino Acid Transporters

PURPOSE AND RATIONALE

Glutamate is not only the predominant excitatory neurotransmitter in the brain but also a potent neurotoxin. Following release of glutamate from presynaptic vesicles into the synapse and activation of a variety of ionotropic and metabotropic glutamate receptors, glutamate is removed from the synapse. This is achieved through active uptake of glutamate by transporters located pre- but also post-synaptically, or glutamate can diffuse out of the synapse and be taken up by transporters located on the cell surface of glial cells. The excitatory amino acid transporters form a gene family out of which at least 5 subtypes were identified (Robinson et al. 1993; Seal and Amara 1999). A role for glutamate transporters has been postulated for acute conditions such as stroke, CNS ischemia, and seizure, as well as in chronic neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis. Glutamate transport is coupled to sodium, potassium and pH gradients across the cell membrane creating an

electrogenic process. This allows transport to be measured using electrophysiological techniques (Vandenberg et al. 1997).

PROCEDURE

Complementary DNAs encoding the human glutamate transporters, EAAT1 and EAAT2, are subcloned into pOTV for expression in *X. laevis* oocytes (Arriza et al. 1994; Vandenberg et al. 1995). The plasmids are linearized with BAMHI and cRNA is transcribed from each of the cDNA constructs with T7 RNA polymerase and capped with 5',7-methyl guanosine using the mMMESSAGE mMACHINE (Ambion, Austin TX). cRNA (50 ng) encoding either EAAT1 or EAAT2 is injected into defolliculated Stage V *X. laevis* oocytes. Two to 7 days later, transport is measured by two-electrode voltage-clamp recording using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) and a MacLab 2e recorder (ADI Instruments, Sydney, Australia) and controlled using a pCLAMP 6.01 interfaced to a Digidata 1200 (Axon Instruments). Oocytes are voltage-clamped at -60 mV and continuously superfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.5). For transport measurement, this buffer is changed to one containing the indicated concentration of substrate and/or blocker. The voltage dependence of block of glutamate transport is measured by clamping the membrane potential at -30 mV and then applying a series of 100 ms voltage pulses from -100 mV to 0 mV and measuring the steady state current at each membrane potential. This protocol is applied both before and during the application of the compound in question and then the base-line current at each membrane potential is subtracted from the current in the presence of the compounds to get a measure for the transport specific current at the various membrane potentials.

EVALUATION

Current (I) as a function of substrate concentration ($[S]$) is fitted by least squares to

$$I = I_{\max}[S]/(K_m + [S])$$

where I_{\max} is the maximal current and K_m is the Michaelis transport constant. The I_{\max} values for the various substrates are expressed relative to the current generated by a maximal dose of L-glutamate in the same cell. I_{\max} and K_m values are expressed as mean \pm standard error and are determined by fitting data from individual oocytes. The potent competitive

blockers are characterized by Schild analysis (Arunlakshana and Schild 1959) and the K_b estimated from the regression plot. The less potent blockers are assumed to be competitive and K_i values calculated from IC_{50} values using the equation

$$K_i = IC_{50}/(1 + [\text{glutamate}]/K_m)$$

where K_i is the inhibition constant, IC_{50} is the concentration giving half maximum inhibition, K_m is the transport constant and $[\text{glutamate}]$ is $30 \mu\text{M}$. The fraction of the membrane electric field sensed by transport blockers when bound to the transporters is estimated using the Woodhull equation (Woodhull 1973),

$$K_i = K_i^0 \exp(-\zeta \delta FE/RT)$$

where K_i is the inhibition constant, K_i^0 is the inhibition constant at 0 mV, ζ is the charge on the blocker, δ is the fraction of the membrane field, F is Faraday's constant, E is the membrane potential, R is the gas constant and T is temperature in K.

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[^{35}S]TBPS Binding in Rat Cortical Homogenates and Sections

PURPOSE AND RATIONALE

To screen potential anticonvulsant agents which interact at the convulsant binding site of the benzodi-

azepine/GABA/chloride ionophore complex by measuring the inhibition of binding of [^{35}S]TBPS to rat cortical membranes.

TBPS, t-butylbicyclophosphorothionate, is a potent convulsant which blocks GABA-ergic neurotransmission by interacting with the convulsant (or picrotoxin) site of the GABA/benzodiazepine/chloride ionophore receptor complex (Casida et al. 1985; Gee et al. 1986; Olsen et al. 1986; Squires et al. 1983; Supavilai and Karabath 1984). Picrotoxin, pentylenetetrazol and the so-called “cage convulsants” are believed to change the state of the chloride channel to a closed conformation, and thereby block GABA-induced increases in chloride permeability. Anticonvulsants such as the barbiturates and the pyrazolopyridines, cartazolate, etazolate and tracazolate, appear to interact at depressant sites allosterically coupled to the convulsant sites, and facilitate the effects of GABA on chloride permeability, by converting the ionophore to the open conformation. Benzodiazepines interact at a separate recognition site to modulate the actions of GABA. Convulsant compounds and some anticonvulsants can inhibit [^{35}S]TBPS binding. These two classes can be differentiated by their effects on dissociation kinetics (Macksay and Ticku 1985; Trifiletti et al. 1984, 1985). [^{35}S]TBPS dissociates slowly, half-life approximately 70 min, in a monophasic manner in the presence of convulsant compounds; anticonvulsants produce a biphasic dissociation, with rapid and slow phase components. It has been postulated that the rapid and slow phases of [^{35}S]TBPS dissociation may correspond to the open and closed conformation of the chloride ionophore.

PROCEDURE

Reagents

- Buffer A 0.05 M Tris with 2 M KCl, pH 7.4
 - 6.61 g Tris HCl
 - 0.97 g Tris Base
 - 149.1 g KCl
 - q.s. to 1 liter with distilled water
- Buffer B
 - a 1:10 dilution of buffer A in distilled water (5 mM Tris, 200 mM KCl, pH 7.4)
- [^{35}S]TBPS is obtained from New England Nuclear with a high initial specific activity, 90–110 Ci/mmol. For an inhibition assay with a 2 nM final concentration of TBPS, a specific activity of 20–25 Ci/mmol will provide sufficient counts due to a high counting efficiency (87%)

for ^{35}S . The specific activity of [^{35}S]TBPS can be reduced with the addition of 3–5 volumes (accurate measurement with a Hamilton syringe) of an equimolar ethanolic solution of non-radio-labeled TBPS (7.9×10^{-6} M). The new specific activity (Ci/mmol) is calculated by dividing the number of Curies by the number of mmoles TBPS. Since [^{35}S]TBPS has a relatively short half-life, 87.1 days, the specific activity is calculated for each assay, based on the exponential rate of decay:

A_0 = initial specific activity

A = specific activity at time t

t = days from date of initial calibration

of specific activity

$t_{1/2}$ = half-life of [^{35}S] in days (87.1)

For IC_{50} determinations, a 40 nM stock solution is made with distilled water and 25 μl is added to each tube to yield a final concentration of 2 nM in the assay.

4. Unlabeled TBPS is available from New England Nuclear. A stock dilution of 7.923×10^{-6} M is prepared in ethanol.
5. Picrotoxin, is obtained from Aldrich Chemical Company. A solution of 5×10^{-4} M is prepared with distilled water, with sonication if necessary. Aliquots of 10 μl are added to assay tubes to give a final concentration of 10^{-5} M.
6. Test compounds. A stock solution of 1 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Whole cerebral cortex of male Wistar rats is dissected over ice and homogenized with a Tekmar Tissumizer, 20 s at setting 40, in 20 volumes of 0.32 M sucrose, ice-cold. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4°C). The supernatant is then centrifuged at 40,000 g for 30 min (SS34, 20,000 rpm, 4°C). The resulting pellet is resuspended in 20 volumes of ice-cold distilled water with two 6-s bursts of the Tissumizer, setting 40. The homogenate is centrifuged at 40,000 g for 30 min. The pellet is washed (resuspended and centrifuged) once with 20 volumes ice-cold buffer (Tris HCl 5 mM, KCl 200 mM, pH 7.4). The resulting pellet is resuspended with 20 volumes buffer and frozen at -70°C overnight. The following day, the tissue homogenate is thawed in a beaker of warm water, approximately 15 min, and then centrifuged at 40,000 g for 30 min (SS34, 20,000 rpm,

4°C). The pellet is washed twice with 20 volumes of ice-cold buffer, and then resuspended and frozen at -70°C for future use. On the day of the assay, the homogenate is thawed and centrifuged at 40,000 g for 30 min. The resulting pellet is washed once with 20 volumes ice-cold buffer and finally resuspended in 30 volumes buffer. Aliquots of 250 μl are used for each assay tube, final volume 500 μl , and correspond to 8.35 mg original wet weight tissue per tube, approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate:

190 μl distilled water

25 μl Tris 0.05 M, KCl 2 M, pH 7.4

10 μl picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor 25 μl [^{35}S]-TBPS, final concentration 2 nM

250 μl tissue preparation, 1:30 homogenate

2. Following the addition of the tissue, the tubes are incubated at 25°C for 150 min with agitation. The assay is terminated by rapid filtration over Whatman GF/B filter circles, presoaked in buffer, with 5×4 ml rinses of ice-cold buffer. Vacuum filtration is performed with the 45-well filtration units to avoid contamination of the Brandell harvesters with [^{35}S]. The filters are counted with 10 ml Liquiscint.

EVALUATION

Specific binding is determined from the difference between binding in the absence or presence of 10 mM picrotoxin, and is typically 85–90% of total binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

Modifications for Dissociation Experiments

1. Prepare assay tubes as follows:

185 μl distilled water

25 μl Tris 50 mM, KCl 2 M, pH 7.4

10 μl test compound or vehicle

2. Add 250 μl tissue homogenate to tubes. Vortex. Pre-incubate 30 min at 25°C.
3. Add 25 μl [^{35}S]-TBPS. Vortex. Incubate 180 min at 25°C.
4. Add 5 μl picrotoxin (10^{-3} M) to give a final concentration of 10^{-5} M. Vortex.

5. At various times after the addition of picrotoxin (0–120 min) tubes are filtered and rinsed as described above.

Modification for [³⁵S]-TBPS Autoradiography

- Sections of rat brain, 20 mm thickness, are collected onto gel-chrome alum subbed slides, freeze-dried for approximately 1 h and stored at -70°C until used.
- After thawing and drying at room temperature, the sections are preincubated for 30 min in buffer B.
- Preparation of slide mailers for incubation:
 - for scintillation counting:
 - 2.47 ml distilled water
 - 0.325 ml buffer A
 - 3.25 ml buffer B
 - 0.13 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
 - 0.325 ml [³⁵S]TBPS, final concentration 2 nM
 - 6.50 ml final volume
 - for autoradiography:
 - 4.56 ml distilled water
 - 0.60 ml buffer A
 - 6.00 ml buffer B
 - 0.24 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
 - 0.60 ml [³⁵S]TBPS, final concentration 2 nM
 - 12.0 ml final volume
- Sections are incubated in slide mailers at room temperature with [³⁵S]TBPS in the absence or presence of appropriate inhibitors for 90 min.
- Slides are transferred to vertical slide holders and rinsed in ice-cold solutions as follows: dip in buffer B, two 5 minute rinses in buffer A and a dip in distilled water.
- Slides are dried under a stream of cool air and desiccated overnight at room temperature.
- Slides are mounted onto boards with appropriate [³⁵S] brain mash standards.
- In the dark room under safelight illumination (GBX filter), slides are opposed to Kodak X-OMAT AR film and stored in cassettes for 7–10 days.
- Develop films as described in “X-OMAT AR Film Processing”.

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| | [³⁵ S]TBPS binding parameters | |
|-------------------------------------------|-------------------------------------------|-----------------------------------------------------------------------------------|
| | Slide-mounted sections | Cortical homogenates |
| Assay conditions | | |
| Tissue | 20 μ sections, rat freeze-dried, 1 h | Whole cortex, rat 1:30 homogenate prepared with 5 washes and 2 freeze-thaw cycles |
| | 30 min preincubation | No preincubation |
| Buffer | 5 mM Tris 200 mM KCl pH 7.4 | 5 mM Tris 200 mM KCl pH 7.4 |
| Incubation time | 90 min, 21–22°C | 150 min, 25°C |
| Non specific | 10^{-5} M picrotoxin | 10^{-5} M picrotoxin |
| Tissue linearity | 2.5–25 mg tissue per 0.5 ml assay tube | |
| Equilibrium constants | | |
| KD (nM) | 32.8 | 25.2 |
| Bmax (fmol/mg prot) | 1615 | 2020 |
| Binding kinetics | | |
| Association kobs (min ⁻¹) | 0.0496 | 0.0138 |
| k+1 (nM ⁻¹ min ⁻¹) | 0.0164 | 0.0021 |
| Dissociation k-1 (min ⁻¹) | 0.017 | 0.001 |
| Dissociation constant k+1/k-1 (nM) | 1.03 | 4.73 |
| IC₅₀M | | |
| Picrotoxin | 2.8×10^{-7} | 3.4×10^{-7} |
| TBPS | 8.7×10^{-8} | 8.1×10^{-8} |
| GABA | 1.7×10^{-6} | 2.1×10^{-6} |
| Pentobarbital | 1.2×10^{-4} | 6.0×10^{-4} |
| Phenobarbital | None at 10^{-3} | None at 10^{-3} |
| Clonazepam | None at 10^{-6} | None at 10^{-6} |

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E.3.1.11

[³H]glycine Binding in Rat Cerebral Cortex

PURPOSE AND RATIONALE

The amino acid glycine is a major inhibitory transmitter in the vertebrate system. Glycinergic synapses are particularly abundant in spinal cord and brain stem, but are also found in higher regions, including the hippocampus. The inhibitory actions of glycine are potently blocked by strychnine. Glycine modulates and may activate the excitatory amino acid receptors of the NMDA-subtype (Thomson 1989; Laube et al. 2002).

The strychnine-sensitive, postsynaptic glycine receptor is a ligand-gated chloride channel protein that belongs to the nicotinic acetylcholine receptor family. It is a pentameric transmembrane protein composed of α and β subunits (Lynch 2004).

Glycine has been shown *in vitro* to potentiate the effects of l-glutamate or NMDA on the stimulation of [³H]TCP binding (Snell et al. 1987, 1988; Bonhaus 1989) and [³H]norepinephrine release (Ransom et al. 1988), and *in vivo* to act as a positive modulator of the glutamate-activated cGMP response in the cerebellum (Danysz et al. 1989; Rao et al. 1990). The activation of NMDA receptors requiring the presence of glycine is necessary for the induction of long-term potentiation (LTP), a type of synaptic plasticity which may be fundamental to learning processes (Oliver et al. 1990). A [³H]glycine binding site in the brain has been identified and characterized as a strychnine-insensitive site associated with the NMDA receptor complex (Kessler et al. 1989; Monahan et al. 1989; Cotman et al. 1987). Autoradiographic studies have shown a similar distribution of [³H]glycine and [³H]TCP (NMDA ion channel radioligand) binding sites (Jansen et al. 1989). Compounds which interact with the glycine site of-

fer a novel mechanism of action for intervention with NMDA receptor function.

Schmieden and Betz (1995) reviewed the pharmacology of the inhibitory glycine receptor, the agonist and antagonist actions of amino acids and piperidine carboxylic compounds.

Hyperekplexia is a hereditary neurological disorder in humans characterized by an excessive startle response which can be caused by mutations in the α_1 -subunit of the heteropentameric inhibitory glycine receptor (Rees et al. 2002). Becker et al. (2002) generated transgenic mice resembling this disease.

The following assay is used to assess the affinity of compounds for the glycine binding site associated with the *N*-methyl-D-aspartate (NMDA) receptor complex using [³H] glycine as the radioligand.

PROCEDURE

Reagents

1. Buffer A: 0.5M Tris maleate, pH 7.4
59.3 g Tris maleate
q.s. to 0.5l
Adjust pH to 7.4 with 0.5M Tris base.
2. Buffer B: 50 mM Tris maleate, pH 7.4
Dilute buffer A 1:10 with distilled water; adjust pH with 50 mM Tris maleate (acid) or 50 mM Tris base.
3. Glycine, 5×10^{-2} M
Dissolve 3.755 mg of glycine (Sigma G7126) with 1.0 ml distilled water. Aliquots of 20 μ l to the assay tube will give a final concentration of 10^{-3} M
4. [³H]Glycine is obtained from New England Nuclear, specific activity 45–50 Ci mmol. For *IC*₅₀ determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 μ l are added to yield a final assay concentration of 10 nM.
5. Test compounds. A stock solution of 5 mM is prepared with a suitable solvent and serially diluted, such that the final concentrations in the assay ranges from 10^{-4} to 10^{-7} M. Higher or lower concentrations may be used, depending on the potency of the compound.
6. Triton-X 100, 10% (v/v) (National Diagnostics, EC-606). A stock solution of Triton-X 100, 10% can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton-X 100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1:15 dilution) is preincubated with an aliquot of the 10% solution to give a final concentration of 0.04% (v/v).

Tissue Preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32M sucrose, 15 volumes

of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. Three cortices are pooled for one preparation. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4°C). The supernatant is centrifuged at 20,000 g (SS34, 12,000 rpm, 4°C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 70, 15 s) and spin at 7600 g (SS34, 8000 rpm 4°C) for 20 min. The pellet is resuspended with 15 volumes of cold distilled water and centrifuged. Discard the supernatant and store the pellet at -70°C.

On the day of the assay, the pellet is resuspended in 15 volumes ice-cold 50 mM Tris maleate, pH 7.4. Preincubate the homogenate with Triton-X in a final concentration of 0.04% (v/v) for 30 min at 37°C with agitation. Centrifuge the suspension at 48,000 g (SS34, 20,000 rpm, 4°C) for 20 min. Wash the pellet an additional 3 times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 25 times the original wet weight.

Assay

1. Prepare assay tubes in quadruplicate.

380 µl distilled water

50 µl buffer A, 0.5 M Tris maleate, pH 7.4

20 µl glycine, 10⁻³ M final concentration, or distilled water or appropriate concentration of inhibitor

50 µl [³H] glycine, final concentration 10 nM

500 µl tissue homogenate.

1000 µl final volume

2. Following the addition of the tissue, the tubes are incubated for 20 min in an ice-bath at 0–4°C. The binding is terminated by centrifugation (HS4, 7000 rpm, 4°C) for 20 min. Aspirate and discard the supernatant. Carefully rinse the pellet twice with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml and add an additional 4 ml scintillation fluid.

EVALUATION

Specific binding is determined from the difference of binding in the absence or in the presence of 10⁻⁴ M glycine and is typically 60–70% of total binding. IC₅₀ values for the competing compound are calculated by log-probit analysis of the data.

MODIFICATIONS OF THE METHOD

Baron et al. (1996), Hofner and Wanner (1997), Chazot et al. (1998) described [³H]MDL 105,519 as a high affinity ligand for the NMDA associated glycine recognition site.

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[³H]strychnine-Sensitive Glycine Receptor

PURPOSE AND RATIONALE

The strychnine-sensitive glycine receptor is a member of the family of ligand-gated ion channel receptors. Within this family, the glycine receptor is most closely related to the GABA-receptor. Like the GABA_A-receptor, the glycine receptor has an inhibitory role, mediating an increase in chloride conductance. However, in contrast to the GABA_A-receptor, the glycine receptor is located mainly in the spinal cord and lower brainstem, where glycine appears to be the major inhibitory neurotransmit-

ter. Purification and molecular cloning has shown that the glycine receptor is an oligomeric transmembrane protein complex composed of three α and two β subunits. The inhibitory actions of glycine are potently blocked by strychnine. In addition to strychnine, the steroid derivative RU5135 (Simmonds and Turner 1985), phenylbenzene- ω -phosphono- α -amino acid (Saitoh et al. 1996), and 5,7-dichloro-4-hydroxyquinoline-3-carboxylic acid (Schmieden et al. 1996) antagonize glycine responses in cultured neurons or cells expressing recombinant glycine receptors.

A glycine receptor agonist may be a potential anti-spastic agent.

PROCEDURE

Male Wistar rats weighing about 200 g are sacrificed. About 220 mg of frozen pons and medulla are homogenized in 2 × 10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1, by an Ultra-Turrax homogenizer. The homogenate is centrifuged for 10 min at 30,000 g at 0–4°C in a refrigerated centrifuge. The pellet is rehomogenized in another 2 × 10 ml portion of the same buffer and recentrifuged as before. This washing procedure is repeated a total of four times. The final pellet is resuspended in 200 vol/g original tissue in ice-cold 50 mM potassium phosphate buffer, pH 7.1, with or without 1000 mM NaCl, and used directly for binding assays.

Binding assays consist of 1 ml tissue homogenate, 50 μ l test solution (water or 5% v/v ethanol/water is used for serial dilutions), 50 μ l water, 5% ethanol/water or glycine solution (40 mM final concentration), and 25 μ l [³H]strychnine working solution, final concentration 2 nM. The samples are mixed well and incubated for 20 min in an ice bath. Free and bound radioactivity are separated by filtration through Whatman GF/C glass fiber filters followed by washing with 2 × 10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1. Tritium on the filters is monitored by conventional scintillation counting in 3 ml Hydroluma. Non-specific binding is binding in the presence of 40 mM glycine and is always subtracted from total binding to give specific binding.

EVALUATION

K_i values are calculated as

$$K_i = (IC_{50}/1 + [K_D])/[L]$$

whereby: IC_{50} are the concentrations that inhibit by 50% the specific binding of [³H]strychnine determined

in two independent experiments using at least 3 concentrations of the agent in duplicate assays, $[L]$ is the concentration of the radioligand, K_D is the affinity constant in the absence or the presence of 1000 mM NaCl.

NaCl shift used for differentiating glycine agonists from glycine antagonists is the ratio K_i 1000 mM NaCl versus K_i 0 mM NaCl.

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E.3.1.13

Electrical Recordings from Hippocampal Slices *In Vitro*

PURPOSE AND RATIONALE

The transverse hippocampal slice has been described as a well-defined cortical structure maintained *in vitro* (Skrede and Westgard 1971). The hippocampus slice has the advantage that each slice may contain all hippocampal structures: The chain of neurons goes from the perforant path to granule cells of the dentate gyrus, through mossy fibres to CA-3 pyramidal cells and then through Schaffer collaterals to CA-1 cells with their axons leaving the hippocampus through the alveus. The pyramidal cells lie close together and can be easily seen and penetrated with fine microelectrodes.

PROCEDURE

Male guinea pigs weighing 300–400 g are anesthetized with ether, the brains removed and the hippocampi dissected. Transverse slices of the hippocampus (300–400 μ m thick) are cut in parallel to the alveolar fibres. After preparation, the slices are submerged in 28°C warm saline which is equilibrated with 95% O₂ and 5% CO₂. After a preincubation period of 2 h, slices are transferred in a Perspex chamber (1.5 \times 4 cm) and attached to the bottom consisting of optically plain glass. The chamber is mounted on an inverted microscope allowing detailed inspection of the excised tissue. The slices are superfused by an approximately 3 mm thick layer of 32°C warm saline. Intracellular recordings are achieved by means of micropipettes with tip diameters of less than 0.5 μ m which are filled with 3 mol/l potassium chloride. Under microscopic control the tips of the micropipettes are placed within the stratum pyramidale and moved by means of a step motor driven hydraulic microdrive. For intracellular injections of drugs, e. g., pentylenetetrazol, via the recording microelectrode, a passive bridge is used. Alternatively, drugs are added to the incubation bath.

EVALUATION

The resting membrane potential and paroxysmal depolarizations are recorded before and after application of drugs.

CRITICAL ASSESSMENT OF THE METHOD

The hippocampal slice has been one of the most useful models for the study of basic mechanisms underlying the epilepsies. The model has also been recommended for screening of putative anticonvulsant drugs.

MODIFICATIONS OF THE METHOD

Harrison and Simmonds (1985) performed quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex consisting of cerebral cortex and corpus callosum.

Tissue culture models of epileptiform activity were described by Crain (1972).

Oh and Dichter (1994) studied the effect of a GABA uptake inhibitor on spontaneous postsynaptic currents in cultured rat hippocampal neurons by the whole cell patch clamp method.

Blanton et al. (1989) described whole cell recordings from neurons in slices of reptilian and mammalian cerebral cortex. Synaptic currents and membrane properties could be studied in voltage and current clamps in cells maintained within their endogenous synaptic currents.

Gähwiler (1988), Stoppini et al. (1991) described methods for organotypic cultures of nervous tissue. Hippocampal slices from 2–23-day old rats were maintained in culture at the interface between air and the culture medium. They were placed on a sterile, transparent and porous membrane and kept in Petri dishes in an incubator. This yielded thin slices which remained 1–4 layers thick and were characterized by a well preserved organotypic organization. Excitatory and inhibitory synaptic potentials could be analyzed using extra- or intracellular recording techniques. After a few days in culture, long-term potentiation of synaptic responses could reproducibly be induced.

Using this method, Liu et al. (1995) studied dopaminergic regulation of transcription factor expression in organotypic cultures of developing striatum of newborn rats.

Stuart et al. (1993) reported the implementation of infrared differential interference contrast video microscopy to an upright compound microscope and a procedure for making patch-pipette recordings from visually identified neuronal somata and dendrites in brain slices.

Bernard and Wheal (1995) described an *ex vivo* model of chronic epilepsy using slices of rat hippocampus previously lesioned by stereotactic injections of kainic acid. Extracellular population spikes were recorded from the stratum pyramidale of CA1 after stimulation by bipolar twisted wire electrodes placed in the stratum radiatum of CA1 area proximally to stratum pyramidale near the recording electrode.

Using hippocampal slices prepared from brain tissue of patients undergoing neurosurgery for epilepsy, Schlicker et al. (1996) showed that the serotonergic

neurons of the human hippocampus are endowed with presynaptic inhibitory autoreceptors.

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E.3.1.14

Electrical Recordings from Isolated Nerve Cells

PURPOSE AND RATIONALE

The use of the cell-attached patch clamp configuration to record action potential currents has shown to have utility in the testing for drug actions on ion channels in excitable cell membranes (Kay and Wong 1986; McLarnon 1990, 1991).

PROCEDURE

Preparation of Cultured Cells

The cultured cells are obtained from the hippocampus or the hypothalamus of rat brain. The isolation of the hippocampal CA1 neurones is performed according to the procedure of Banker and Cowan (1977). The dissociated hypothalamic neurons are prepared according to Jirikowski et al. (1981). The hippocampal and hypothalamic neurons that are selected for electrophysi-

ological recording are bipolar in shape with the long axis dimension between 10–15 μ m. The neurons are studied over a period of 5–10 days after isolation.

Electrophysiology

The cell-attached patch clamp configuration is used to record spontaneous action potentials in the cultured neurons. The bath solution contains 140 mM NaCl, 5 mM KCl, 0.5 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.3. The composition of the patch pipette solution is the same as the bath solution. The drugs used in the experiments are added to the bath solution.

The patch pipettes (Corning 7052 glass) are fabricated using a specific patch pipette puller (PP-83; Narishige, Tokyo) are fire-polished and filled immediately prior to use. The resistance of the pipettes is in the range 4–8 M Ω and the tip diameters are between 1 and 2 μ m. An axopatch amplifier (Axon Instruments, Foster City, CA), with low-pass filter set at 5 kHz, is used to record the capacitive currents. After recording, at a sampling frequency of 5 kHz, the data are stored on hard disc or video tape for subsequent analysis. All data are obtained at room temperature (21–24°C).

EVALUATION

The capacitive component of current recorded by the patch pipettes is proportional to the rate of change of membrane potential and can be expressed as $I_C = CdV/dt$, where C is the specific membrane capacitance. Assuming a value of C of 1 μ F/cm² and a tip diameter of the patch pipette of 2 μ m, the membrane area isolated by the patch pipette is about 3×10^{-8} cm². Using a value of dV/dt of 100 mV/ms gives an approximate expected magnitude of I_C near 3 pA. When a class III antiarrhythmic drug that blocks a delayed rectifier K⁺ channel is added to the bath, the portion of I_C corresponding to the after-hyperpolarization component of the action potential is completely abolished. The Na⁺ spike is not altered by the drug. The cell-attached recordings of I_C can also be used to determine effects on the Na⁺ spike when tetrodotoxin is included in the bath solution. Thus, the spontaneous action potential can be used for evaluation of drug effects on both K⁺ and Na⁺ channels in excitable membrane.

MODIFICATIONS OF THE METHOD

Chen et al. (1990) measured current responses mediated by GABA_A receptors in pyramidal cells acutely dissociated from the hippocampus of mature guinea pigs according to the procedure of Kay and Wong (1986) using whole-cell voltage-clamp recordings.

Caulfield and Brown (1992) studied inhibition of calcium current in NG108–15 neuroblastoma cells by cannabinoid receptor agonists using whole-cell voltage-clamp recordings.

Gola et al. (1992, 1993) performed voltage recordings on non-dissociated sympathetic neurones from rabbit coeliac ganglia using the whole-cell configuration of the patch clamp technique (Neher and Sakmann 1976; Sakmann and Neher 1983).

Stolc (1994) used the voltage-clamp technique in internally dialyzed single neurones isolated from young rat sensory ganglia to study the effects of pyridindole stobadine on inward sodium and calcium currents and on slow non-inactivating components of potassium outward current.

McGivern et al. (1995) examined the actions of a neuroprotective agent on voltage dependent Na⁺ currents in the neuroblastoma cell line, NIE-115, using the whole cell variant of the patch clamp technique.

Smith (1995) reviewed the use of patch- and voltage clamp procedures to study neurotransmitter transduction mechanisms.

Using whole-cell and perforated-patch recordings, Delmas et al. (1998) examined the part played by endogenous G-protein $\beta\gamma$ subunits in neurotransmitter-mediated inhibition of N-type Ca²⁺ channel current in dissociated rat superior cervical sympathetic neurones.

Gonzales et al. (1985) registered membrane potentials with intracellular electrodes in cultured olfactory chemoreceptor cells.

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E.3.1.15

Isolated Neonatal Rat Spinal Cord

PURPOSE AND RATIONALE

The spinal cord of the neonatal rat is a useful *in vitro* preparation, originally proposed by Otsuka and Konishi (1974). In this preparation, ventral root potentials of ten seconds of duration can be recorded after supramaximal electrical stimulation of the lumbar dorsal root. Various implicated in the generation of these slow ventral root potentials are tachykinins, such as substance P and neurokinin B (Yanagisawa et al. 1982; Akagi et al. 1985; Otsuka and Yanagisawa 1988; Guo et al. 1998) and agonists at the glutamate receptor sites (Evans et al. 1982; Ohino and Warnick 1988 1990; Shinozaki et al. 1989; Ishida et al. 1990, 1991, 1993; Woodley and Kendig 1991; Bleakman et al. 1992; King et al. 1992; Thompson et al. 1992; Zeman and Lodge 1992; Pook et al. 1993; Jane et al. 1994; Boxall et al. 1996). These long lasting reflexes are thought to reflect a nociceptive reflex for several reasons; the threshold of activation corresponds to that of C fibre primary afferents (Akagi et al. 1985); they can be depressed by opioids (Yanagisawa et al. 1985; Nussbaumer et al. 1989; Faber et al. 1997) and α_2 -adrenoceptor agonists (Kendig et al. 1991) and a similar response can be evoked by peripheral noxious stimulation (Yanagisawa et al. 1995).

PROCEDURE**Preparation of Spinal Cord**

Male Wistar rats aged 6–9 days are used. Under ether anesthesia the spinal column is quickly removed from the animal and placed in a Petri dish, filled with oxygenated physiological solution. A laminectomy is performed on the dorsal surface of the spinal column at room temperature. The spinal cord of the mid-thoracic to mid-sacral level is then carefully removed from the column and hemisected in the longitudinal plane under a dissecting microscope. After removal of the dura mater, the hemisected cord is completely submerged in the recording chamber (total volume: approximately 0.5 ml), which is perfused with physiological solution (124 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 15 mM NaHCO₃, 11 mM glucose) at a flow rate of 1.5 to 2.5 ml/min. The perfusion medium is continuously bubbled with a gas mixture of 95% O₂ and 5% CO₂ and the temperature is kept at 25 ± 0.5°C. The cut ends of the corresponding dorsal and ventral roots in an L_{3–5} segment are fixed to a pair of suction electrodes for stimulating and recording. The preparation is stabilized in the recording chamber for at least 90 min to allow recovery from the dissection and the sealing of the roots to suction electrodes.

Recording of Monosynaptic Reflexes

Test stimulations, composed of square wave pulses of 0.05–0.2 ms duration and 5–30 V, are applied to the dorsal root every 10 s. The discharges of the corresponding ventral root are recorded with a suction electrode, amplified and monitored on an oscilloscope and stored on an analog data recorder or computer discs for later analysis. The mean values for the waveform of the monosynaptic reflex (amplitude, area and latency) are obtained from 6–18 successive responses in each experiment before and during application of drugs.

Recording of Single Motoneuron Activity

Test pulses (0.01–0.1 ms duration and 5–15 V) are applied to the dorsal or ventral root every 2 s. The activity of single motoneurons is recorded extracellularly using glass microelectrodes (electrical resistance approximately 10–30 MΩ) filled with 3 M sodium chloride or 2 M sodium acetate. The microelectrode is inserted into the ventral part of the cord through the hemisected surface while monitoring the field potential. The motoneurons in the ventral horn are identified by the short and consistent latency of antidromic spikes (1.66 ± 0.46 ms, *n* = 5), following the stimulation of the ventral root. The motoneurons also produce

transsynaptic spikes with orthodromic stimulation of the dorsal root, of which the latency is 10.26 ± 1.05 ms upon supramaximal stimulation. The spike generation of motoneurons is displayed on an oscilloscope and stored on magnetic tapes. The spontaneous firing of the motoneuron is also monitored on an oscilloscope and recorded through a window discriminator and spike counter. The mean number and latency of spikes and latency of the dorsal root-elicited spikes are obtained from 20–40 successive responses in each experiment. Comparisons are made before and 3–5 min after application of drugs.

EVALUATION

All data are expressed as the mean ± SEM. Statistical significance of the data is determined by repeated measures analysis of variance (ANOVA) and, when appropriate, Student's *t*-test. A *P* value of less than 0.05 is considered statistically significant.

MODIFICATIONS OF THE METHOD

Smith and Feldman (1987), Wong et al. (1996) described an *in vitro* neonatal rat brainstem/spinal cord preparation. The brainstem and cervical spinal cord were isolated from 0–4 days old ether anesthetized Sprague Dawley rats. The en bloc neuraxis was pinned down with ventral surface upward in a recording chamber and superfused continuously with artificial cerebrospinal fluid. Respiratory activity was recorded with suction electrodes from the C₄ ventral root.

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E.3.1.16

Cell Culture of Neurons

PURPOSE AND RATIONALE

Cell culture of neurons, especially of hippocampal neurons, has become a widely used tool in pharmacological studies (Banker and Cowan 1977; Skaper et al. 1990, 1993, 2001; Araujo and Cotman 1993; Brewer 1997, 1999; Brewer et al. 1998; Li et al. 1998; Mitoma et al. 1998; Semkova et al. 1998, 1999; Chaudieu and Privat 1999; May et al. 1999; Hampson et al. 2000; Novitskaya et al. 2000; Pickard et al. 2000; Vergun et al. 2001).

The basic information on methodology of cell culture of rat hippocampal neurones was given by Banker and Cowan (1977). One modification used by Skaper et al. (1990, 2001) studying the role of mast cells on potentiation by histamine of synaptically mediated excitotoxicity in cultured hippocampal neurones is described below.

PROCEDURE

Preparation of Hippocampus

Timed pregnancies are obtained in female Sprague Dawley rats by daily checking vaginal washings for sperm, the day on which sperm is found being regarded as day 0. At the appropriate stage of gestation the pregnant rats are anesthetized and the uterus removed to a sterile dish. The remainder of the cell preparations is performed in a sterile hood.

The brains are removed from the fetuses with a pair of fine scissors, and the cerebral hemispheres separated from the brain stem. When the hemisphere of an 18- to 19-day-old fetus is viewed in a dissecting microscope the hippocampus can be clearly seen on its medial surface. The hippocampal fissure, usually marked by a conspicuous group of blood vessels, indicates the approximate junction between the hippocampus and the adjoining subicular and entorhinal cortex. The developing fimbria is seen as a white translucent band along the free margin of the hippocampus. Before separating the hippocampus from the hemisphere, the meninges

and adherent chorioid plexus are carefully pulled off with fine forceps. At this stage the full depth of the hippocampal fissure can be seen. Then with iridectomy scissors the hippocampus is separated from the adjoining cortex by a cut parallel to the hippocampal fissure, and by transverse cuts at its rostral and caudal ends.

Cell Culture

Hippocampi isolated from embryonic rats (gestational age 17.5 days) are incubated with 0.08% trypsin, and dissociated in Neurobasal medium containing 10% heat-inactivated calf serum. Cells are pelleted by centrifugation (200 g, 5 min) and resuspended in Neurobasal medium containing B27 (Life Technologies, Inc.) supplements (with antioxidants), 25 μ M glutamate, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. The cell suspension is plated onto poly-D-lysine (10 μ g/ml) coated 48-well culture plates at a density of 4.5×10^4 cells per cm^2 . Cultures are maintained at 37°C in a humidified atmosphere of 5% CO₂-95% air. After 5 days, one-half of the medium is replaced with an equal volume of maintenance medium (plating medium but containing B27 supplements without antioxidants, and lacking glutamate). Additional medium exchanges (0.5 volume) are performed every 3–4 days thereafter. Cells are used between 14 and 16 days in culture. During this period, neurones develop extensive neuritic networks, and form functional synapses.

Mast cells are collected from the peritoneal lavage of male Sprague Dawley rats and isolated over a bovine serum albumin gradient to >90% purity, as judged by toluidine blue and safranin staining.

Neurotoxicity Assays

Cultures are washed once with Locke's solution (pH 7.0–7.4) with or without 1 mM MgCl₂. Drug treatments are carried out for 15–30 min (25°C) in a final volume of 0.5 ml. In the case of mast-cell-neurone co-cultures, transwell inserts (3- μ m pore size, 9 mm diameter) are seeded with 5×10^4 mast cells in RPMI-1640 medium and placed in 24-well plates overnight. Inserts with mast cells are then placed into wells with hippocampal cells. Mast cells activation is achieved using an antigenic stimulus (0.3 μ g/ml anti-DNP IgE/0.1 μ g/ml DNP albumin). The mast cell containing inserts are removed at the end of the Mg²⁺-free incubation. After this time all cell monolayers are washed with complete Locke's solution and returned to their original culture medium for 24 h. Cytotoxicity is evident during 24 h after the insult. Vi-

able neurons have phase-bright somata of round-to-oval shape, with smooth, intact neurites. Neurones are considered nonviable when they exhibit neurite fragmentation and somatic swelling and vacuolation. Cell survival is quantified 24 h after the insult by a colorimetric reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

EVALUATION

Data are analyzed by one-way ANOVA with Student-Newman-Keuls *post hoc* test for differences between groups.

MODIFICATIONS OF THE METHOD

Brewer (1997) reported the isolation and culture of adult rat hippocampal neurons. Using different proteases and special separation techniques, about 90000 viable neurons could be isolated from each hypothalamus at any age rat from birth to 24–36 months. Neurons were cultured for more than 3 weeks.

Flavin and Ho (1999) found that propentofylline protects hippocampal neurons in culture from death triggered by macrophage or microglia secretory products.

To study neurite outgrowth in cultured hippocampal cells from Wistar rat embryos, 5000 cells/well were seeded in 8-well LabTec tissue culture slides with a grown surface of permanox plastic and grown in Neurobasal medium supplemented with B27 (Life Technologies, Inc.), 20 mM HEPES, 0.4% bovine serum albumin, penicillin (100 IU/ml), and streptomycin (100 μ g/ml). (Novitskaya et al. 2000). For image analysis, cells were fixed in 4% paraformaldehyde and stained for 20 min with Coomassie Blue R250. Cover slides were observed in an inverted microscope using phase contrast optics. To measure neurite outgrowth from hippocampal neurons, an unbiased counting frame containing a grid with a number of test lines was superimposed on the images of cells. The number of intersections of cellular processes with the test lines was counted and related to the number of cell bodies, thereby allowing quantification of neurite length per cell.

Cell culture experiments were also performed with **neuronal cells from other areas of the brain** besides the hippocampus.

Brain tissue samples of rat embryos containing either septum plus preoptic area or retrochiasmatic hypothalamus were dissociated and cultured for 14 and 21 days by Jirikowski et al. (1981). By means of immunofluorescence, LHRH, α -MSH, vasopressin and neurophysin-containing hormones could be identified.

Sinor et al. (2000) studied NMDA and glutamate evoked excitotoxicity at distinct cellular locations in rat cortical neurones *in vitro*.

Canals et al. (2001) examined neurotrophic and neurotoxic effects of nitric oxide on neuronal enriched fetal midbrain cultures from embryonic Sprague Dawley rats.

López et al. (2001) investigated the release of amino acid neurotransmitters in cultured cortical neurons obtained from gestation day 19 rats by nicotine stimulation.

Ehret et al. (2001) studied the modulation of electrically evoked acetylcholine release in cultured septal neurones from embryonic Wistar rats.

Tang et al. (2001) found a lack of replicative senescence in cultured rat oligodendrocyte precursor cells.

Yamagishi et al. (2001) used cultured rat cerebellar granule neurons as a model system for studying neuronal apoptosis.

Noh and Koh (2000) prepared mixed **mouse** cortical cultures containing both neurons and astrocytes, and pure astrocyte cultures, from fetal (15 d of gestation) and neonatal (1–3 postnatal days) mice.

Saluja et al. (2001) found that PPAR δ agonists stimulate oligodendrocyte differentiation in glial cell culture of mouse cerebra.

Ushida et al. (2000) succeeded to directly isolate clonogenic **human** central nervous system stem cells from fresh human brain tissue, using antibodies to cell surface markers and fluorescence-activated cell sorting.

For further studies with brain cell cultures see Sect. F.2.0.10.

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E.3.2

In Vivo Methods

E.3.2.1

Electroshock in Mice

PURPOSE AND RATIONALE

The electroshock assay in mice is used primarily as an indication for compounds which are effective in grand mal epilepsy. Tonic hindlimb extensions are evoked by electric stimuli which are suppressed by anti-epileptics but also by other centrally active drugs.

PROCEDURE

Groups of 6–10 male NMRI mice (18–30 g) are used. The test is started 30 min after i.p. injection or 60 min after oral treatment with the test compound or the vehicle. An apparatus with corneal or ear electrodes (Woodbury and Davenport 1952) is used to deliver the stimuli. The intensity of stimulus is dependent on the apparatus, e. g., 12 mA, 50 Hz for 0.2 s have been used. Under these conditions all vehicle treated mice show the characteristic extensor tonus.

EVALUATION

The animals are observed closely for 2 min. Disappearance of the hindleg extensor tonic convulsion is used as positive criterion. Percent of inhibition of seizures relative to controls is calculated. Using various doses, ED_{50} -values and 95% confidence interval are calculated by probit analysis.

ED_{50} -values after oral administration are:

- Diazepam 3.0 mg/kg
- Diphenylhydantoin 20.0 mg/kg

CRITICAL ASSESSMENT OF THE METHOD

The electroshock test in mice has been proven to be a useful tool to detect compounds with anticonvulsant activity.

MODIFICATIONS OF THE METHOD

Cashin and Jackson (1962) described a simple apparatus for assessing anticonvulsant drugs by the electroshock seizure test in mice.

Kitano et al. (1996) developed the increasing-current electroshock seizure test, a new method for assessment of anti- and pro-convulsant activities of drugs in mice. A single train of pulses (square wave, 5 ms, 20 Hz) of linearly increasing intensity from 5 to 30 mA was applied via ear electrodes. The current at which tonic hindlimb extension occurred was recorded as the seizure threshold. The method allows the determination of seizure threshold current for individual animals.

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E.3.2.2**Pentylentetrazol Test in Mice and Rats**

See Sect. E.2.2.1.

E.3.2.3**Strychnine-Induced Convulsions in Mice**

See Sect. E.2.2.2.

E.3.2.4**Picrotoxin-Induced Convulsions in Mice**

See Sect. E.2.2.3.

E.3.2.5**Isoniazid-Induced Convulsions in Mice**

See Sect. E.2.2.4.

These tests, already described for evaluation of the anti-convulsive activity of anxiolytics, can be used and show activity for anti-epileptics.

Many other agents induce seizures in animals and have been used to test the anticonvulsant activity of drugs (Stone 1972), e. g., glutarimides (Hahn and Oberdorf 1960), pilocarpine (Tursky et al. 1987), methionine sulfoximine (Toussi et al. 1987), *N*-methyl-D-aspartic acid (Leander et al. (1988), γ -hydroxybutyrate (Snead 1988).

Shouse et al. (1989) described mechanisms of seizure suppression during rapid-eye-movement (REM) sleep in cats. Spike-wave paroxysms in the EEG accompanied by bilateral myoclonus of the head and the neck were induced by i.m. injection of 300000 to 400,000 IU/kg sodium penicillin G.

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E.3.2.6**Bicuculline Test in Rats****PURPOSE AND RATIONALE**

Seizures can be induced by the GAGA_A-antagonist bicuculline and are antagonized by known anti-epileptics.

PROCEDURE

Female Sprague-Dawley rats are injected i.v. with 1 mg/kg bicuculline. At this dose, a tonic convulsion appears in all treated rats within 30 s after injection. Test compounds are administered orally 1 or 2 h before bicuculline injection. Dose-response curves can be obtained.

EVALUATION

Percentage of protected animals is evaluated. *ED*₅₀-values and 95% confidence limits are calculated by probit analysis.

CRITICAL ASSESSMENT OF THE METHOD

Like the electroshock test, the bicuculline test is considered to be relatively specific for anti-epileptic activity.

MODIFICATIONS OF THE METHOD

Czuczwar et al. (1985) studied the antagonism of *N*-methyl-D,L-aspartic acid-induced convulsions by anti-epileptic drugs and other agents.

REFERENCES AND FURTHER READING

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E.3.2.7

4-Aminopyridine-Induced Seizures in Mice

PURPOSE AND RATIONALE

The K⁺ channel antagonist 4-aminopyridine is a powerful convulsant in animals and in man. The drug readily penetrates the blood-brain barrier and is believed to induce seizure activity by enhancing spontaneous and evoked neurotransmitter release. Although both excitatory and inhibitory synaptic transmission is facilitated by 4-aminopyridine, the epileptiform activity induced by the drug is predominantly mediated by non-NMDA type excitatory amino acid receptors. In mice, parenterally administered 4-aminopyridine induces clonic-tonic convulsions and lethality.

PROCEDURE

Male NIH Swiss mice weighing 25–30 g are allowed to acclimatize with free access to food and water for a 24-h period before testing. Test drugs are administered in various doses intraperitoneally 15 min prior to s.c. injection of 4-aminopyridine at a dose of 13.3 mg/kg which was found to be the LD₉₇ in this strain of mice. Controls treated with 4-aminopyridine only exhibit characteristic behavioral signs, such as hyperreactivity, trembling, intermitted forelimb/hindlimb clonus followed by hindlimb extension, tonic seizures, opisthotonus and death. The mean latency to death at the LD₉₇ is about 10 min. Groups of 8 mice are used for each dose.

EVALUATION

The percentage of protected animals at each dose is used to calculate ED₅₀ values. Phenytoin-like anticonvulsants such as carbamazepine and broad spectrum anticonvulsants such as phenobarbital and valproate are effective whereas GABA-enhancers such as diazepam, several NMDA antagonists, and CA²⁺ channel antagonists such as nimodipine are not.

CRITICAL ASSESSMENT OF THE METHOD

The profile of drugs effective in this seizure model is distinct from other chemoconvulsant models and more similar to those that prevent tonic hindlimb extension

in the maximal electroshock seizure test. The test is useful to differentiate the mode of action of anticonvulsant drugs.

MODIFICATIONS OF THE METHOD

Morales-Villagran et al. (1996) described protection against seizures induced by intracerebral or intracerebroventricular administration of 4-aminopyridine by NMDA receptor antagonists.

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E.3.2.8

3-Nitropropionic Acid-Induced Seizures in Mice

PURPOSE AND RATIONALE

3-Nitropropionic acid is a naturally occurring toxin demonstrated to impair energy metabolism via irreversible inhibition of a mitochondrial complex II component, succinate dehydrogenase (Alston et al. 1977; Ludolph et al. 1991). 3-Nitropropionic acid evokes seizures in mice after i.p. injection of 100–200 mg/kg (Urbańska et al. 1998, 1999). Urbańska et al. (1998) and Zuchora et al. (2005) evaluated anticonvulsants for their protective effect against 3-nitropropionic-acid-induced seizures.

PROCEDURE

Male albino Swiss mice weighing 20–25 g were injected i.p. with 210 mg/kg 3-nitropropionic acid, which is equal to the ED₉₇ dose (i. e., the dose required to evoke seizures in 97% of the animals). Groups of eight mice received in addition various doses of the anticonvulsant drugs. Percentage of animals with seizures and latency until occurrence of seizures were determined. Mortality rate was determined 2 h after injection of 3-nitropropionic acid.

EVALUATION

ED₅₀ and LD₅₀ values together with their confidence limits were estimated by computerized fitting of the data by linear regression analysis according to Litchfield and Wilcoxon. Statistical comparisons of latency data were performed by means of one-way analysis of variance (ANOVA) followed by adjustment of *P* value by the Bonferroni method.

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E.3.2.9

Epilepsy Induced by Focal Lesions

PURPOSE AND RATIONALE

Intrahippocampal injections of noxious agents or certain cerebral lesions can induce seizures in animals. Cavalheiro et al. (1982) studied the long-term effects of intrahippocampal kainic acid injections in rats.

PROCEDURE

Adult male Wistar rats are anesthetized with a chloral hydrate/Nembutal mixture and placed in a stereotactic apparatus. For injections, a 0.3 mm cannula is inserted through a burr hole in the calvarium. The coordinates for hippocampal injections are based on a stereotactic atlas, e.g., Albe-Fessard et al. (1971). Kainic acid is dissolved in artificial serum and infused in various doses (0.1 to 3.0 µg) in a volume of 0.2 µl over a period of 3 min. For recording, bipolar twisted electrodes (100 µm) are positioned stereotaxically and fixed on the skull with dental acrylic cement. Depth recording sites include the dorsal hippocampus and amygdala ipsilateral to the injected side. Surface electrodes are guided from jeweler's screws over the occipital cortex. An additional screw in the frontal sinus serves as indifferent electrode for grounding. Signals are recorded by an EEG polygraph.

EVALUATION

EEG recordings and observations of convulsive seizures are performed during the acute phase and during the chronic phase (up to 2 months) with and without drug treatment.

MODIFICATIONS OF THE METHOD

Several agents have been used as convulsants after topical administration, e.g., application of alumina cream (Kopeloff et al. 1942, 1955; Ward 1972; Feria-Velasco et al. 1980), implantation of cobalt powder (Dow et al. 1962; Fischer et al. 1967), injection of a colloidal gel of tungstic acid (Blum and Liban 1960; Black et al. 1967), topical application of penicillin (Matsumoto and Marsan 1964), subpial injection of saturated FeCl₃ solution (Reid et al. 1979; Lange et al. 1980), intracerebral injections of zinc sulfate (Pei et al. 1983), intracerebral injection of antibodies to brain gangliosides (Karpiak et al. 1976, 1981), microinjections of cholinergic agonists (Ferguson and Jasper 1971; Turski et al. 1983), topical application of atropine (Daniels and Spehlman 1973), injection of tetanus toxin into the hippocampus (Mellanby et al. 1984; Hawkins and Mellanby 1987), injection of strychnine in the visual or somato-sensory cortex (Atsev and Yosiphov 1969), electrophoretic application of bicuculline from a fluid filled microelectrode (Campbell and Holmes 1984).

Bernhard et al. (1955, 1956) evaluated the anticonvulsive effect of local anaesthetics in cats and monkeys. The head was fixed in light Nembutal anaesthesia, the parietal areas exposed and covered with paraffin oil. Stimulating electrodes were placed at the surface of the parietal region. The cortex was stimulated with repetitive square wave shocks (duration 1–3 ms) with a frequency of 25 per s for 5 s. In order to avoid muscular movements, d-tubocurarine was given. Cortical afterdischarge was registered before and after injection of local anaesthetics.

Cortical epileptic lesions were produced by local freezing (Stalmaster and Hanna 1972; Hanna and Stalmaster 1973; Loiseau et al. 1987).

Repetitive electrical stimulation of discrete regions of the central nervous system has been used as a convenient method for reproduction of the ictal phenomena of epilepsy (Marsan 1972; Racine 1972).

Remler and Marcussen (1986), Remler et al. (1986) studied the pharmacological response of systemically derived focal epileptic lesions. A defined area of left hemisphere of rats was radiated by α-particles from a cyclotron destroying the blood brain barrier. After a period of 150 days following irradiation, bicu-

culline was injected intraperitoneally resulting in focal lesions with EEG spikes and convulsions. Anticonvulsant drugs decreased these effects.

Walton and Treiman (1989) and Walton et al. (1994) described a model of cobalt-lesioned rats in which status epilepticus was induced by injection of homocysteine thiolactone.

Anderer et al. (1993) pointed out that restriction to a limited set of EEG-target variables may lead to misinterpretation of pharmacology-EEG results.

Krupp and Löscher (1998) developed a cortical ramp-stimulation model allowing repeated determinations of seizure threshold at short time intervals in individual rats without inducing postictal threshold increases.

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E.3.2.10**Kindled Rat Seizure Model****PURPOSE AND RATIONALE**

Kindling, first described by Goddard et al. (1969), results from repetitive subconvulsive electrical stimulation of certain areas of the brain. Initially, local after-discharge is associated with mild behavioral signs; however, with continued stimulation electrical activity presumably spreads, and generalized convulsions occur. Although the pathogenesis of kindled seizures is not fully understood, it serves as a useful tool for investigating the efficacy of experimental anticonvulsant agents.

PROCEDURE

Adult female Sprague-Dawley rats (270–400 g) are used. The rats are implanted with an electrode in the right amygdala according to the coordinates of Pellegrino et al. (1979): frontal, 7.0; lateral, –4.7; horizontal, 2.5. At least 1 week has to elapse before electrical stimulation of the brain is started. After discharge threshold is determined for each rat. Duration and amplitude, behavioral seizure duration and seizure stage are recorded with increased stimuli afterdischarges. Seizure severity is classified into 5 stages (Racine 1972). Rats are considered to be kindled on the first stimulation causing a stage 5 seizure which is followed by at least 2 consecutive stage 5 seizures.

The animals are tested on the day before and after treatment with the test compound (i.p. or orally). Amygdala stimulation is applied at various time intervals.

EVALUATION

The occurrence and the degree of seizures are compared between control results and the those after administration of the test compound.

CRITICAL ASSESSMENT OF THE METHOD

The kindled seizure model offers an approach to study anticonvulsive drugs on the basis of a pathophysiological model. This method may give more relevant results than the simpler methods using maximal electroshock or chemically induced convulsions.

MODIFICATIONS OF THE METHOD

Generalized convulsive seizures have been induced by daily amygdaloid stimulation in **baboons** (Wada and Osawa 1976), and in **rhesus monkeys** (Wada et al. 1978).

The kindling effect can be produced by intermittent administration of small doses of pentylenetetrazol (Mason and Cooper 1972).

Dürmüller et al. (1994) tested a competitive (NBQX) and a non-competitive (GYKI 52446) AMPA antagonist, and a competitive NMDA antagonist (D-CPPene) against the development of kindling and against fully kindled seizures in amygdala-kindled rats.

Croucher et al. (1996) described a chemical kindling procedure in rats by daily focal microinjection of NMDA into the right basolateral amygdala and the inhibition of seizures by a NMDA receptor antagonist.

Suzuki et al. (1996) studied the anticonvulsant action of metabotropic glutamate receptor agonists in kindled amygdala of rats.

Löscher et al. (1993), Ebert et al. (1997), and Ebert and Löscher (1999) studied the effect of phenytoin on the spread of seizures in the amygdala kindling model in rats. Sprague Dawley rats implanted with a stimulation and recording electrode in the basolateral amygdala showed an increase in current intensity necessary for eliciting after-discharges of about 200% after administration of phenytoin, while seizure severity at threshold was increased compared to controls. Phenytoin-resistant kindled rats are considered as a model of drug-resistant epilepsy.

Löscher (1998) discussed the pharmacology of glutamate receptor antagonists in the kindling model of epilepsy.

The kindling procedure can also be used to evaluate antidepressant drugs (Babington 1975).

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E.3.2.11

Posthypoxic Myoclonus in Rats

PURPOSE AND RATIONALE

The syndrome of posthypoxic myoclonus in man was described by Lance and Adams (1963). Lance (1968), Fahn (1986). Troung et al. (1994), Jaw et al. (1994, 1995, 1996) reported on a model in rats resembling this human disorder.

PROCEDURE

Male Sprague Dawley rats fasted 12–24 h prior surgery are anesthetized with 100 mg/kg ketamine i.p., supplemented by 0.4 mg/kg atropine. The animal is placed on a circulating water pad and kept at a constant body temperature by a heating lamp. The rat is intubated and ventilated with 30% O₂ in N₂O. A femoral artery and vein are cannulated for monitoring blood pressure and delivery of drugs, respectively. Electrocardiogram and blood pressure are recorded with a polygraph. The rat is then paralyzed with 2 mg/kg succinylcholine i.v. and ventilator settings are adjusted to a rate of 60 strokes/min and a volume of 7.5 ml/kg, which yields blood gases of >150 mm Hg pO₂, 35–40 mm Hg pCO₂, and a pH of 7.35–7.40. N₂O is replaced with N₂ and an equilibrium period of 5 min is allowed.

Cardiac arrest is accomplished with a trans-thoracic intracardiac injection of KCl and cessation of the respiration. Resuscitation is begun 10 min after the arrest by turning on the ventilator (100% O₂), manual thoracic compressions, and i.v. injections of 20 µg/kg epinephrine hydrochloride and sodium bicarbonate (4 mEq/kg). The rat is then weaned from the ventilator over 2–4 h and extubated.

Auditory-induced myoclonus: Rats are presented with a series of 45 clicks from a metronome (1 Hz, 95 dB, 40 ms), and the response to each click is scored as follows: 0 = no response, 1 = ear twitch, 2 = ear and head jerk, 3 = ear, head, and shoulder jerk, 4 = whole body jerk, 6 = whole body jerk of such severity that it causes a jump. The total myoclonus score of each rat is determined by summing up the scores yielded over 45 clicks.

Since rats ranging from 3 to 14 days post cardiac arrest show similar susceptibility to audiogenic stimulation, animals within this period are used for pharmacological tests. Myoclonus scores are assessed 30 min before and 60 min after intraperitoneal drug application.

EVALUATION

Changes in myoclonus scores are analyzed by paired two-tailed Student's *t*-test.

CRITICAL ASSESSMENT OF THE TEST

Some anticonvulsant drugs, such as clonazepam and valproic acid were reported to be active in this test; however, phenytoin is not. Posthypoxic myoclonus may present a special pathological condition.

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E.3.2.12

Rat Kainate Model of Epilepsy

PURPOSE AND RATIONALE

Temporal lobe epilepsy is characterized by complex partial seizures that involve and apparently originate in the mesial temporal structures of the limbic system. These complex partial seizures can evolve into secondarily generalized, tonic-clonic seizures. Patients become resistant to the treatment with the usual anti-epileptic drugs. The kainate-treated rat is one of several models used to study temporal lobe epilepsy. Examination of the hippocampus and dentate gyrus from kainate-treated rats has revealed a similar pattern of neurodegeneration in the hippocampus and the presence of mossy fiber sprouting in the inner molecular level of the dentate gyrus. Several authors used this

model to find drugs for treatment-resistant epilepsy (Bolanos et al. 1998; Hellier et al. 1998; Longo and Mello 1998; Maj et al. 1998; Bouillere et al. 1999; Pitkänen et al. 1999; Cilio et al. 2001; Madsen et al. 2001; Ebert et al. 2002; Tamagami et al. 2004). Maj et al. (1998) tested the activity of several drugs against kainate-induced status epilepticus and hippocampal lesions in the rat.

PROCEDURE

Male Wistar rats weighing 225–250 g are anesthetized with sodium pentobarbital (50 mg/kg i.p.). They are implanted extradurally with electrodes over the frontal and parietal cortex and with a reference electrode on the cerebellum. Caution is taken not to break the inner table of the diploe. All the electrodes are connected to plugs and held to the skull with dental acrylic cement. At least 7 days after surgery, rats are treated with either saline or test drugs intraperitoneally. Then, 15 min later, the rats receive a single i.p. dose of kainic acid (10 mg/kg). EEG recordings and behavioral observations are performed up to 240 min after kainic acid administration. Status epilepticus is defined as a sustained ictal EEG pattern lasting 20 min or longer without any interruption longer than 1 min.

Seven days later, the rats are sacrificed, the brains removed and immersed for 48 h in 10% formalin. Coronal sections (4 μm) are stained with hematoxylin-eosin. Hippocampal injury is assessed by counting the number of histologically normal CA4 pyramidal neurons.

EVALUATION

The percentage of animals protected from status epilepticus is analyzed using Fisher's exact test. For calculation of the latency to status epilepticus (min) and duration of status epilepticus (min), all animals are included regardless of whether they showed status epilepticus or not. The data are evaluated by analysis of variance (ANOVA) followed by Dunnett's test. Neuronal counts are analyzed using the Mann-Whitney non-parametric test.

MODIFICATIONS OF THE METHOD

Cilio et al. (2001) used immature rats to test the anticonvulsant action and long-term effects of gabapentin.

Hellier et al. (1998) used repeated low-dose systemic treatment in order to reduce the mortality associated with single injections with kainate.

Since intracerebroventricular administration of kainic acid decreases hippocampal neuronal number

and increases dopamine receptor binding in the nucleus accumbens, kainic lesions have been discussed as an animal model of schizophrenia (Bardgett et al. 1995; Csernansky et al. 1998).

Humphrey et al. (2001) described methods for inducing neuronal loss in preweanling rats using an intracerebroventricular infusion of kainic acid.

Hu et al. (1998) investigated neuronal stress and injury in C57/BL mice after systemic kainic acid administration.

Bouilleret et al. (1999) tested recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice.

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E.3.2.13

Pilocarpine Model of Epilepsy

PURPOSE AND RATIONALE

Several post-status models are described in which epilepsy develops after a chemically induced status epilepticus, such as the kainate, the pilocarpine and the pilocarpine-lithium model (Löscher 2002). Several modifications of the pilocarpine and the lithium-pilocarpine model are reported in the literature (Cavalheiro et al. 1991; Leite and Cavalheiro 1995; André et al. 2001; Biagini et al. 2001; Klitgaard et al. 2002; Leite et al. 2002; Wallace et al. 2003; Arida et al. 2004; Leroy et al. 2004; Lyon et al. 2004; Rigoulot et al. 2004; Setkowicz et al. 2004). When rats are pretreated with lithium chloride, status epilepticus can be produced with a substantially lower dose of pilocarpine, and rats display the same clinical and EEG features of status epilepticus as with pilocarpine alone (Honchar et al. 1983). André et al. (2001) and Rigoulot et al. (2004) tested antiepileptic drugs in the lithium-pilocarpine model of epilepsy.

PROCEDURE

Male Wistar rats weighing 225–250 g were anesthetized for electrode implantation by an i.p. injection of 2.5 mg/kg diazepam and 1 mg/kg ketamine chlorhydrate. Two single-contact recording electrodes were placed on the skull, one on each side of the parietal cortex, and one bipolar deep recording electrode was placed in the right hippocampus (Vergnes et al. 1982).

One week after surgery, rats received 3 mEq/kg lithium chloride i.p. On the following day, 1 mg/kg methylscopolamine bromide was administered s.c. to limit the peripheral effects of the convulsant. Status epilepticus was induced by injecting pilocarpine (25 mg/kg s.c.) 30 min after methylscopolamine. Various doses of test drug (i.p.) or 2.5 mg/kg diazepam (i.m.) were injected at 1 h after the onset of status epilepticus. The onset of status epilepticus corresponds to the moment at which rats experience successive seizures without recovery. Continuous spiking of the

EEG occurs 30–60 min after pilocarpine administration. The bilateral EEG cortical activity and the unilateral EEG hippocampal activity were recorded during the whole duration of status epilepticus and concurrent behavioral changes were noted.

Quantification of neuronal damage was performed 14 days after status epilepticus. Brains of rats sacrificed in pentobarbital anesthesia were removed and coronal sections containing the hippocampus from the anterior to the posterior level were prepared. Quantification of cell density was performed with a microscopic grid. The numbers of cells obtained in 12 counted fields were averaged.

EVALUATION

Statistical analysis of neuronal damage and epilepsy between the different groups was performed by means of analysis of variance followed by a posthoc Dunnett's test for multiple comparisons.

MODIFICATIONS OF THE METHOD

Hort et al. (1999) studied the relation between spontaneous recurrent seizures and the derangement of cognitive function in pilocarpine-induced status epilepticus,

Tang et al. (2004) recorded EEG in freely moving mice after pilocarpine-induced status epilepticus. A transmitter (TSE Systems, Bad Homburg, Germany) was fixed on the electrode socket by plug connection with wires attached to the skull by two screws 3 days before pilocarpine induction. The EEG signals were telemetrically received via an HF receiver which passed the signals to the computer.

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E.3.2.14

Self-Sustained Status Epilepticus

PURPOSE AND RATIONALE

Status epilepticus causes neuronal damage that is associated with cognitive impairment. Self-sustained status epilepticus (SSSE) can be induced in rats by electrical stimulation of the perforant pathway (Halonen et al. 1996, 1999, 2001; Pitkänen et al. 1996; De Vasconcelos et al. 1999; Mazarati et al. 1999, 2004). This model is used to find antiepileptic drugs for patients with therapy-resistant epilepsy. Pitkänen et al. (1996), Halonen

et al. (1996, 1999, 2001), and Mazarati et al. (2004) studied the effect of drugs on status epilepticus in rats.

PROCEDURE

Under ketamine (60 mg/kg) and xylazine (15 mg/kg) anesthesia, male Wistar rats weighing 260–280 g were implanted with a bipolar stimulation electrode into the angular bundle of the perforant path (0.5 mm anterior and 4.5 mm left to lambda) and a bipolar recording electrode into the ipsilateral dentate gyrus (3 mm posterior and 2.5 mm left to bregma). The depth of the electrode was 3.5–4 mm from the brain surface and was optimized by finding the maximal population spike evoked from the dentate gyrus by stimuli applied to the perforant path.

For induction of self-sustained status epilepticus perforant path stimulation was delivered using a Grass stimulator model 8800, for 30 min with the following parameters: 10-s, 20-Hz trains for 1 ms, 30-V pulses delivered every minute, together with continuous 2 Hz stimulation using the same parameters.

Test drugs were injected i.v. into the tail vein either 20 min before perforant path stimulation, or 10 or 40 min after the end of perforant path stimulation. Control animals were treated with saline.

Electrographic activity was acquired and analyzed off-line using Harmonie software (Stellate Systems, Montreal), configured for automatic detection and saving spikes and seizures. Analysis of EEG was performed by a “blinded” unbiased investigator. All seizure EEGs were reviewed manually.

EVALUATION

The following indices were used to quantify seizure activity: duration of self-sustained status epilepticus (= time between the end of perforant path stimulation and the end of the last electrographic seizure); cumulative seizure time (the sum of the duration of all individual seizures); number of seizure episodes; average duration of individual seizures (cumulative seizure time divided by number of seizures); number of spikes per hour. Statistical analysis was carried out with one-way ANOVA followed by Newman-Keuls post hoc test, or, if the normality test failed, ANOVA on ranks followed by Mann-Whitney post hoc test.

MODIFICATIONS OF THE METHOD

Brown et al. (1953) and Barton et al. (2001) characterized the 6 Hz psychomotor seizure model of partial epilepsy in rats.

Nissinen et al. (2000) described a model of chronic temporal lobe epilepsy induced by electrical stimulation of the lateral nucleus of the amygdala in rats.

Walton et al. (1996) induced status epilepticus in rats with actively epileptogenic cortical cobalt lesions by administration of homocysteine thiolactone.

Laurén et al. (2003) described selective changes in gamma-aminobutyric acid type A receptor subunits in the hippocampus in spontaneously seizing rats with chronic temporal lobe epilepsy.

Brandt et al. (2003) studied epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats.

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E.3.2.15

Rat Model of Cortical Dysplasia

PURPOSE AND RATIONALE

Epilepsy becomes drug resistant in 20%–30% of patients. Cortical dysplasia is implicated as a major contributing factor of many types of epileptic disorders that are resistant to pharmacological intervention (Becker 1991; Aicardi 1994). Several animal models of cortical dysplasia with specific clinical pathologies have been described (Amano et al. 1996; Jacobs 1996; Jacobs et al. 1999; Lee et al. 1997; Hirotsune et al. 1998; Chevassus au Louis et al. 1999; Zhu and Roper 2000; Wenzel et al. 2001; Benardete and Kriegstein 2002; Morimoto et al. 2004; Jacobs and Prince 2005).

Baraban and Scharzhkroin (1995, 1996), Baraban et al. (2000), and Smyth et al. (2002) exposed rats *in utero* to methylazoxymethanol (MAM).

PROCEDURE

Dysplastic and control rats were generated by injecting pregnant Sprague Dawley rats on day 15 of gestation with 25 mg/kg i.p. MAM or vehicle (10% DMSO in 0.3 ml 0.9% saline).

For *in vitro* studies, recordings were performed using acute hippocampal slices from adult vehicle or MAM-treated rats. Hippocampi were not dissected out, and all slices included entorhinal cortex and other overlying cortical structures. After cutting, slices remained submerged in a holding chamber containing oxygenated recording medium (nACF) consisting of (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 2 CaCl₂ and 10 dextrose. A slice was then transferred to a gas-interface recording chamber and perfused with oxygenated nACF at a flow rate of 2.5 ml/min at 33.5°C. Borosilicate glass electrodes were pulled, filled with 2 M NaCl (2–8 MΩ) and placed in the CA1 region of stratum pyramidale and/or within neuronal heterotopias under visual microscopic control. A mono-polar stimulating electrode was placed in stratum radiatum. Voltage was recorded with a Neurodata IR-283 amplifier and monitored on a PC running pClamp software. Spontaneous field activity and evoked population spikes were stored on hard disk for later blinded analysis. Interictal epileptiform burst activity was initiated with perfusion of nACF containing 4-aminopyridine (100 μM), a potassium channel blocker known to cause seizures in hu-

mans and spontaneous epileptiform activity in hippocampal slice preparations. The 4-aminopyridine *in vitro* seizure model is based on blockade of A-type potassium channels leading to the appearance of giant excitatory postsynaptic potentials generated by the prolonged firing of pyramidal neurons in CA3 burst-generating regions of hippocampus. Burst frequency was determined by counting the number of interictal epileptiform events per second during a 3-min epoch before and after 60 min of antiepileptic drug co-perfusion, and was expressed as Hz. Burst amplitude (1.5–6 mV) was determined by measuring the average peak-to-peak interval for ten consecutive representative bursts during the same epoch. Evoked synaptic responses were analyzed by averaging the number of population spikes obtained on ten consecutive sweeps recorded after stratum radiatum stimulation (0.3- to 3-mA pulses 100 μs pulse-width). A downward voltage deflection ≥ 0.5 mV superimposed on the population excitatory post-synaptic potential (EPSP) was defined as a “population spike”; the number of population spikes was compared for each slice during perfusion with normal ACSF (baseline), ACSF plus 4-aminopyridine and ACSF plus 4-aminopyridine and antiepileptic drug. For each slice experiment, the population spike was monitored every 15 min.

For *in vivo* studies, control and MAM-exposed rats were administered 15 mg/kg kainic acid, a concentration that reliably produces acute seizure activity. Behavioral activity was scored on a 6-stage scale (Germano and Sperber 1997). Animals were treated with 400 mg/kg i.p. valproate 30 min before kainate injection. Latencies to the first sign of hyperexcitability and to the first tonic-clonic seizure were recorded.

EVALUATION

Date were plotted graphically as “survival” curves, and differences in mean latencies were ranked and analyzed using a non-parametric Kruskal-Wallis one-way ANOVA.

CRITICAL ASSESSMENT OF THE TEST

Since the MAM-exposed rats exhibit a dramatically reduced sensitivity to commonly prescribed antiepileptic drugs, this model is considered to be relevant for drug-resistant epilepsy.

MODIFICATIONS OF THE METHOD

Léré et al. (2002) described a model of “epileptic tolerance” for investigating neuroprotection, epileptic susceptibility and gene expression-related plastic changes. Expression of status epilepticus was triggered

by infusion of the excitotoxic agent kainate in the right hippocampus of adult rats. An appropriate dose of kainate was used to cause brain damage to the homolateral, but not contralateral, hippocampus. At various times following the preconditioning insult, kainite was then re-administered into the lateral ventricle and neuroprotection was observed in the contralateral side between 1 and 15 days later.

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E.3.2.16

Genetic Animal Models of Epilepsy

PURPOSE AND RATIONALE

Several animal species exhibit epilepsy with spontaneous recurrent seizures such as dogs, rats, and mice (Löscher 1984). Serikawa and Yamada (1986) described spontaneous epileptic rats which are double mutants and exhibit both tonic and absence-like seizures.

PROCEDURE

Spontaneous epileptic rats are obtained by mating the tremor heterozygous rat (*tm/+*) with the zitter homozygous rat (*zi/zi*) found in a Sprague-Dawley colony. The behavior of the spontaneous epileptic rats is recorded weekly for 2 h on videotapes. The frequency of tonic convulsions and wild jumping occurring in the absence of external stimuli are recorded. Under anesthesia silver ball-tipped and monopolar stainless-steel electrodes are chronically implanted in the left frontal cortex and hippocampus. An indifferent electrode is placed on the frontal cranium. The frequency of absence-like seizures and tonic convulsions, as well as the duration of each seizure, are measured on the EEG. A mild tactile stimulus is given on the back of the animal every 2.5 min to induce consistent tonic convulsions. Compounds are given i.p. or orally.

EVALUATION

The number of seizures and the duration of each seizure are obtained and the total duration of the seizures (number × duration) is calculated every 5 min before and after injection of the drug. Percent changes between values before and after drug administration are calculated.

CRITICAL ASSESSMENT OF THE TEST

Studies in spontaneous epileptic rats and other genetic models are of value for an in depth investigation of a potential anti-epileptic drug.

MODIFICATIONS OF THE METHOD

The **tremor rat** (*tm/tm*) was described as a model of petit mal epilepsy (Serikawa and Yamada 1986;

Serikawa et al. 1987). Seki et al. (2002) attempted to determine whether gene transfer of aspartoacylase inhibited absence-like seizures in tremor rats using recombinant adenovirus. Noda et al. (1998) and Iida et al. (1998) described the **NER rat strain** (Noda epileptic rat) as a genetic model in epilepsy research, which was developed by inbreeding rats with spontaneous tonic-clonic seizures in a stock of Crj:Wistar.

The **genetic epileptic WAG/RiJ rat** has been recommended as a useful model for general absence epilepsy in humans (Van Luijtelaar and Coenen 1986; Coenen et al. 1992; Budziszewska et al. 1999; Van Luijtelaar et al. 2003; Sarkisova et al. 2003; Bouwman and van Rijn 2004). Danober et al. (1995, 1998), Deransart et al. (2000), Lakaye et al. (2002), and Nehling and Boehrer (2003) studied the **GAERS rat**, the genetic absence epilepsy rat from Strasbourg, which shows generalized non-convulsive absence seizures characterized by the occurrence of synchronous and bilateral spike and wave discharges.

Amano et al. (1996) developed an **epileptic rat mutant with spontaneous limbic-like seizures** by successive mating and selection from an inherited cataract rat.

Racine et al. (1999) reported selective breeding of **kindling-prone** and kindling-resistant **rats**. The selection of these strains was based on their rates of amygdala kindling. From a parent population of Long Evans hooded and Wistar rats, the males and females that showed the fastest and slowest amygdala kindling rates were selected and bred.

Sarkisian et al. (1999) described seizures in the **Flathead (FH) rat** as a genetic model in early postnatal development.

Tsubota et al. (2003) identified the **Wakayama epileptic rat (WER)** in a colony of Wistar rats, a mutant exhibiting both tonic-clonic seizures and absence-like seizures

Several other genetic animal models have been described (Löscher and Frey 1984; Löscher and Meldrum 1984) showing epilepsy with spontaneous recurrent seizures, such as:

dogs (Cunningham 1971; Edmonds et al. 1979),

rats with petit mal epilepsy (Vergnes et al. 1982), rats with two mutations, zitter and tremor (Serikawa and Yamada 1986; Xie et al. 1990), rats with absence-like states and spontaneous tonic convulsions (Sasa et al. 1988),

tottering mice (Green and Sidman 1962; Noebels 1979; Noebels and Sidman 1979; Fletcher et al. 1966; Tehrani et al. 1997),

leaner mutant mice with severe ataxia and atrophic cerebellum (Herrup and Wilczynski 1982; Heckroth and Abbott 1994),

the quaking mouse (Sidman et al. 1966; Chermat et al. 1981) having deficiencies in myelination in the nervous system (Hogan 1977; Li et al. 1993; Bartoszewicz et al. 1995) and alterations in the dopaminergic (Nikulina et al. 1995) and α_2 -adrenergic (Mitrovic et al. 1992) brain system,

the stargazer mutant mouse which shows generalized non-convulsive spike wave seizures with behavioral arrest that resembles the clinical phenotype of general absence epilepsy (Noebels et al. 1990; Di Pasquale et al. 1997) with a disrupted *Cacng2* gene (Letts et al. 2005),

the lethargic (lh/lh) mouse as a model of absence seizures (Hosford et al. 1999).

There are models of **epilepsy with reflex seizures**, such as:

baboons with photomyoclonic seizures (Killam et al. 1966, 1967; Stark et al. 1970; Naquet and Meldrum 1972; Killam and Killam 1984; Smith et al. 1991; Chapman et al. 1995),

photosensitive fowls (Crawford 1969, 1970),

the Fayoumi strain of chickens (Fepi) (Batini et al. 2004),

audiogenic seizure susceptible mice (Collins 1972; Seyfried 1979; Chapman et al. 1984; Stenger et al. 1991),

mechanically stimulated mice (Imaizumi et al. 1959; Oguro et al. 1991),

the EL mouse which is a strain highly susceptible to convulsive seizures after repeated sensory stimulation (Seyfried et al. 1986; King and LaMotte 1989; Green and Seyfried 1991; Wang et al. 1997; Suzuki 2004),

audiogenic seizure susceptible rats (Wistar Audiogenic Rats WAR) (Consroe et al. 1979; Reigel et al. 1986; Smith et al. 1991; Patel et al. 1990; Scarlatelli-Lima et al. 2003; Galvis-Alonzo et al. 2004; Magalhães et al. 2004).

The genetically epileptic-prone rat GEPR

responding to acoustic stimulation has been described by Ko et al. (1982), Dailey and Jobe (1985), Dailey et al. (1989), Faingold et al. (1988), Faingold et al. (1992, 1994), Jobe et al. (1992, 1995) and Laird (1989). The inferior colliculus is strongly implicated as a critical initiation

site within the neuronal network for audiogenic seizures. Two strains were characterized: **GEPR-3** exhibiting moderate or clonic convulsions, and **GEPR-9** exhibiting more severe tonic extensor convulsions (Dailey et al. 1996; Kurtz et al. 2001; Moraes et al. 2005).

Gerbils with reflex seizures were described by Thiessen et al. (1968); Loskota et al. (1974); Majkowski and Kaplan (1983); Lee and Lomax (1984), Bartoszyk and Hamer (1987), and Lee et al. (1987).

Löscher et al. (1989) discussed the **sz mutant hamster** as a genetic model of epilepsy or of paroxysmal dystonia.

Quesney (1984) reported **generalized photosensitive epilepsy in cats** after long-term intramuscular administration of low-dose penicillin.

Famula et al. (1997) and Oberbauer et al. (2003) described the epidemiology of epilepsy in **tervurens (Belgian shepherd dogs)**; Srenk et al. (1994) in **Golden Retrievers**.

Seizure susceptibility was described in **Drosophila** (Kuebler and Tanouye 2000; Kuebler et al. 2001; Zhang et al. 2002).

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E.3.2.17**Transgenic Animals as Models of Epilepsy****PURPOSE AND RATIONALE**

The availability of transgenic animals has stimulated research on pathogenesis of epilepsy. Several surveys on this topic are available (Allen and Walsh 1999; Meldrum et al. 1999; Noebels 1999; Prasad et al. 1999; Toth and Tecott 1999; Schauwecker 2002; Weinschenker and Szot 2002; Upton and Stratton 2003; Giorgi et al. 2004; Yang and Frankel 2004). Several studies contribute to the understanding of pathology of epilepsy (Butler et al. 1995; Zeng et al. 1997; Campbell et al. 2000; Liang et al. 2000; Musumeci et al. 2000; Viswanath et al. 2000; Kearney et al. 2001; Knuesel et al. 2002; Potschka et al. 2002; Shimizu et al. 2002; Ludwig et al. 2003; Ferri et al. 2004; Diano et al. 2005; Peters et al. 2005).

Lüthi et al. (1997) found that mutant mice overexpressing protease nexin-1 (PN-1) in brain under the control of the Thy-1 promoter (Thy 1/PN-1) or lacking PN-1 (PN-1 $-/-$) develop epileptic activity *in vivo*. An endogenous serine protease inhibitor modulated epileptic activity and hippocampal long-term potentiation.

Kunieda et al. (2002) recommended mice with systemic overexpression of the alpha 1B-adrenergic receptor as an animal model of epilepsy.

Some of the studies gave hints for further development of antiepileptic drugs, such as the neuropeptide galanin (Kokaia et al. 2001; Mazarati et al. 2004) or the neuropeptide-Y (Shannon and Yang 2004). Several studies were devoted to the role of brain-derived neurotrophic factor (BDNF) (Lahtinen et al. 2002, 2003, 2004).

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E.4 Hypnotic Activity

E.4.0.1

General Considerations

The term “hypnotic” has to be defined. In man, the purpose of taking hypnotics is to obtain a “normal” night’s sleep from which the patient can be aroused without any subsequent hangover. In animal experiments, the term “hypnotic” has been applied to a much deeper stage of central depression of drug induced unconsciousness associated with loss of muscle tone and of righting reflexes. Therefore, most of the pharmacological models are questionable in regard to their predictivity to find an ideal hypnotic for human therapy. Many of the pharmacological tests are based on the potentiation of sleeping time induced by barbiturates or other sedative agents.

Since the biochemical events during sleep are rather unknown no *in vitro* method exists for testing compounds with potential hypnotic activity.

E.4.1

In Vivo Methods

E.4.1.1

Potentiation of Hexobarbital Sleeping Time

PURPOSE AND RATIONALE

The test is used to elucidate CNS-active properties of drugs. Not only hypnotics, sedatives, and tranquilizers but also antidepressants at high doses are known to prolong hexobarbital induced sleep after a single dose of hexobarbital. The loss of righting reflex is measured as criterion for the duration of hexobarbital-induced sleeping time. Mice are used in this test, since metabolic elimination of hexobarbital is rapid in this species.

PROCEDURE

Groups of 10 male NMRI-mice with an average weight of 18–22 g are used. They are dosed orally, i.p. or s.c. with the test compound or the reference standard (e. g., 3 mg/kg diazepam p.o.) or the vehicle. Thirty min after i.p. or s.c. injection or 60 min after oral dosing 60 mg/kg hexobarbital is injected intravenously. The animals are placed on their backs on a warmed (37°C) pad and the duration of loss of the righting reflex (starting at the time of hexobarbital injection) is measured until they regain their righting reflexes. Injection of 60 mg/kg hexobarbital usually causes anesthesia for about 15 min. If there is any doubt as to the reappearance of the righting reflex, the subject is placed gently on its back again and, if it rights itself within one minute, this time is considered as the endpoint.

EVALUATION

Mean values of duration of anesthesia (min) are recorded in control and experimental groups. The percent change in duration of anesthesia is calculated in the experimental groups as compared to those of the controls. ED_{50} values can be calculated. ED_{50} is defined as the dose of drug leading to a 100% prolongation in duration of anesthesia in 50% of the animals.

CRITICAL ASSESSMENT OF THE METHOD

The anxiolytic agents of the benzodiazepine type show an uniform pattern with oral ED_{50} values of less than 1 mg/kg. This is in agreement with the fact that barbiturates also show anxiolytic activity in anti-anxiety

tests with animals as well as in patients. Neuroleptics, such as chlorpromazine and haloperidol, also prolong hexobarbital sleeping time in low doses. The test is considered to be unspecific since compounds which inhibit liver metabolism of hexobarbital also prolong time of anesthesia. Balazs and Grice (1963) discussed the relationship between liver necrosis, induced by CCl_4 or nitrosamines, and pentobarbital sleeping time in rats.

OTHER USES OF THE TEST

Hexobarbital sleeping time is not only prolonged by the simultaneous administration of many compounds but also shortened under special conditions. Several CNS-active compounds (analeptics and stimulants like amphetamine and related compounds and methylxanthines) reduce hexobarbital sleeping time. Standard compounds for this kind of procedure are pentylenetetrazol, methamphetamine and aminophylline.

After repeated administrations, induction of metabolic enzymes in the liver is caused by many compounds and leads to an increased destruction of hexobarbital. Due to the accelerated metabolism of hexobarbital, sleeping time is reduced (Remmer 1972).

MODIFICATIONS OF THE TEST

Instead of hexobarbital, another barbiturate, thiopental can be used which has been proven in clinical use to be a short acting anesthetic. Test compounds or the standard are given 60 min before i.v. injection of 25 mg/kg thiopental to mice with a weight between 18 and 22 g. The animals are placed on their backs and the reappearance of the righting reflex is observed. The ED_{50} which results in a 100% prolongation in duration of anesthesia is between 2.5 and 4.0 mg/kg diazepam p.o.

Simon et al. (1982) tested the interaction of various psychotropic agents with sleep induced by barbital or pentobarbital in mice. Pentobarbital (50 mg/kg) or barbital (180 mg/kg) were injected i.p. and the latency and duration of sleep (loss of righting reflex) were recorded. The test compound was usually administered i.p. 30 min before the injection of the barbiturate. The test was recommended for detecting sedative or anti-sleep activity. Since pentobarbital is metabolized by the liver whereas barbital is not, a comparative study using the two compounds can be useful for determining whether an eventual potentiation or antagonism can be ascribed to enzymatic inhibition or induction.

Fujimori (1965) recommended the use of barbital-Na instead of hexobarbital for the sleeping time test

since barbital is not biotransformed by the liver microsomal system.

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E.4.1.2

Experimental Insomnia in Rats

PURPOSE AND RATIONALE

James and Piper (1978) described a method for evaluating potential hypnotic compounds in rats. Usually, the compounds are tested in normal animals where they do not significantly decrease wakefulness. Footshock induced “insomnia” in rats is proposed as suitable model for insomnia in patients.

PROCEDURE

Male Wistar rats (200–275 g) are prepared for chronic electroencephalographic and electromyographic recordings. Four silver/silver chloride epidural electrodes and two disc nuchal electrodes are implanted. A minimum of 10 days is allowed for recovery from surgery. The animals are placed into sound-attenuated recording chambers with grid floors. The frontal-occipital electroencephalogram and the electromyogram are recorded via nonrestraining recording leads on a polygraph and a tape recorder.

On the control day, the animals are dosed with the vehicle and a control nonstress recording is obtained for 8 h. On the next day, the animals are again injected with the vehicle and then exposed to electric footshocks for 8 h. The footshock is delivered through the grid floor of the recording chamber using the EMG

leads as indifferent electrodes, in the form of a 0.5 mA pulse of 15 ms width for 30 s at 1 Hz. During the foot-shock the EEG and EMG recording circuits are automatically interrupted. The delivery of electric foot-shock is triggered automatically by two adjustable timers. In this way, each shock period of 30 s is followed by an interval of 30 min. On the next day the rats are dosed with the test compound or the standard and recordings are obtained during a shock session of 8 h.

EVALUATION

The sleep-wake cycle is definitely altered by the stress procedure. The amounts of arousal and of slow wave sleep I are increased, whereas slow wave sleep II and paradoxical sleep are decreased. Phenobarbital and benzodiazepines antagonize these changes at least partially.

CRITICAL ASSESSMENT OF THE METHOD

For screening procedures, the method is too expensive and time-consuming. However, the EEG-parameters in a situation of insomnia similar to men can indicate the usefulness of a new compound.

MODIFICATIONS OF THE METHOD

Gardner and James (1987) described a modified shortened protocol in which a 2.5-h nonstressed control period is followed by drug or vehicle administration and a further 5.5-h recording of the electrocorticogram in the presence of intermittent footshock.

Laval et al. (1991) studied the effect of anxiolytic and hypnotic drugs on sleep circadian rhythms in the rat.

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E.4.1.3

EEG Registration in Conscious Cats

PURPOSE AND RATIONALE

The effect of hypnotics on sleep pattern of EEG tracings can be studied in conscious, freely moving

cats with chronically implanted electrodes (Heinemann et al. 1970, 1973; Wallach et al. 1976; Hirotsu et al. 1988).

PROCEDURE

Female cats weighing 2.5–3.5 kg are anesthetized and prepared with bipolar subcortical electrodes in the reticular formation (A3, L3, H –1), dorsal hippocampus (A5, L –5, H8), and either amygdala (A12, L –9, H –5), or caudate nucleus (A11, L9.5, H –2). Cortical screw electrodes are placed over the anterior suprasylvian, lateral, medial suprasylvian and ectosylvian gyri. Two Teflon coated steel wires are placed in the cervical neck muscles. All wires are connected to a sub-miniature socket and implanted in dental acrylic. Cats of this chronic colony are then intermittently utilized for drug experiments at interdrug intervals of at least two weeks.

On experimental days, the cats are taken into an experimental chamber 70 × 80 × 80 cm high. The box is lighted and ventilated with room air at 21°C. The cat is immediately connected to a cable which exits through the top center of the cage into a mercury swivel. This prevents the cable from becoming twisted and restricting the cat's movement. Recordings of the cortical EEG, cervical neck muscle tone and reticular formation multiple unit activity are obtained. Continuous recordings for up to 96 h are amplified and stored in a recorder. The recordings of cortical EEG, cervical neck muscle tone and reticular formation multiple unit activity are analyzed for REM sleep, slow wave sleep, and wakefulness. Undefined periods which can not be identified either as slow wave sleep or as wakefulness are included in the awake total. Since a first night effect was observed (Wallach et al. 1996) drugs are given at the third or fourth day.

EVALUATION

The data are analyzed by analysis of variance with subjects, days, and drug as factors.

MODIFICATIONS OF THE METHOD

Schallek and Kuehn (1965) measured the effects of benzodiazepines on spontaneous EEG and arousal responses in cats with implanted electrodes.

In addition to EEG and electromyogram, Holm et al. (1991) registered the electro-oculogram in conscious cats.

EEG studies in immobilized cats were performed by Ongini et al. (1982) for evaluation of a benzodiazepine hypnotic. Adult mongrel cats of both sexes were anesthetized with halothane. A tracheal cannula

was inserted and artificial respiration was maintained throughout the experiment. The spinal cord was transected at C₂ level (Encephalè isolè preparation). The femoral vein was cannulated for i.v. injection of drugs. Cortical electrodes were inserted into the skull in the frontal, parietal and occipital areas. All incisions were infiltrated with mepivacaine 1% to produce local anesthesia. The body temperature was maintained at 36.5–38.0°C by an electrical heat pad. After recovery from surgery and anesthesia, a continuous EEG recording of 2 h was taken prior to drug administration. Test drugs were injected intravenously at various doses. Electrocortical activity was recorded using a 8-channel electroencephalograph. In addition, two electrodes were connected with an EEG-analyzer for the on-line evaluation of the EEG power spectrum. This was computed by the Fast Fourier Transform at a frequency range of 0–32 Hz. Power spectral plots averaging 30 s of electrocortical activity were derived during the experiment.

Shibata et al. (1994) administered various local anesthetics intravenously with constant rates of equipotent doses to cats with implanted electrodes until EEG seizures appeared. During slow rates of infusion, a tetraphasic sequence of changes was found.

Wetzel (1985) evaluated EEG recordings in freely moving **rats** by visual analysis for wakefulness, slow wave sleep or paradoxical sleep.

Krijzer et al. (1991) presented a subclassification of antidepressants based on the quantitative analysis of the electrocorticogram in the rat.

Sarkadi and Inczeffy (1996) described an integrated quantitative electroencephalographic system for pharmacological and toxicological research in the rat. Peak latencies and amplitudes of visual-evoked potentials, occurrence, duration, and linear excursions of photically evoked afterdischarges, activity, mobility, complexity according to Hjorth (1970), and absolute spectral powers of delta, theta, alpha, and beta frequency bands of background activity of visual cortex and frontal-visual leads were measured in freely moving rats.

Rinaldi-Carmona et al. (1992) performed temporal EEG analysis of the sleep-waking cycle in rats with implanted electrodes after administration of a 5-hydroxytryptamine₂ receptor antagonist.

Lozito et al. (1994) compared loss of righting with EEG changes in rats with implanted electrodes after single and multiple infusions of fentanyl analogues.

Jones and Greufe (1994) described a quantitative electroencephalographic method in **dogs**.

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E.4.1.4

Automated Rat Sleep Analysis System

PURPOSE AND RATIONALE

Ruigt et al. (1988a, b, 1993) described an automated rat sleep classification system in rats which allows classification of psychotropic drugs such as potential antidepressants, antipsychotics and stimulants (Ruigt and von Proosdij 1990; de Boer and Ruigt 1995).

The system records and analyzes bioelectrical signals from several animals over extended periods of time. The analysis is based on 3 signals, the parieto-occipital EEG, nuchal EMG and a movement indicator signal.

PROCEDURE

Epidural screw electrodes are implanted over the parieto-occipital cortex of male rats weighing 250–300 g for the recording of EEG against a frontal electrode. Stainless-steel wire electrodes are inserted in the dorsal neck musculature for recording the electromyogram (EMG). After recovery from surgery animals are separately housed in a light- (12:12h light-dark cycle) and temperature- (21°C) controlled room. Twenty-nine hour EEG and EMG recordings are made in sound-attenuated Faraday cages from 32 rats simultaneously. Movements of the rats are detected as capacitive artefacts generated in an open-ended wire of the non-restraining flat cable connecting the rats to a swivel commutator and to amplification and A/D conversion units, which are hooked up through a data controller to

a dedicated PDP-11/83 minicomputer system for on-line spectral EEG analysis and data compression.

Off-line sleep staging on a micro VAX is done per 2-s epoch based on 5 spectral EEG band values (1.0–3.0, 3.0–6.0, 6.0–9.0, 9.5–20.0, 20.0–45.0 Hz), the integrated EMG level and the movement level. A first sleep stage assignment per epoch is done by application of a discriminant function to these epoch values. The discriminant function is derived from a discriminant analysis of visually classified representative recording segments from different sleep stages recorded during a separate calibration experiment for each rat. A moving average EEG smoothing procedure and a set of syntactic classification rules are then used to give a final sleep stage assignment to each specific EEG epoch.

Six sleep-wake stages are distinguished including 2 waking stages: (1) active waking characterized by movement, theta activity and high EMG, and (2) quiet waking without movement. Four sleep stages are discriminated: (3) quiet sleep, characterized by EEG spindles; (4) deep slow-wave sleep with prominent delta activity; (5) pre-REM sleep with spindles against a background of theta activity and low EMG, and (6) REM sleep with theta activity and low EMG.

Each experiment consists of 32 rats divided over maximally 4 groups, including various drug treatment groups (generally several doses of the same drug) and always one placebo group. Drug administration is done at the beginning of the light cycle of the rats. After each experiment 2–3 weeks are allowed for wash-out. Drug effects on sleep-waking behavior are assessed on several parameters extracted from the hypnogram, among which percentage time spent in each of the sleep stages per 30-min period and per rat. This gives for each compound a profile of changes over sleep stages and over time.

EVALUATION

Sleep stage-dependent and sleep-independent parts of the EEG power spectrum are defined by a procedure originally developed by Fairchild et al. (1969, 1971, 1975). First, a normal canonical discriminant analysis is done on 4 EEG frequency bands (1–3, 3–6, 6–9, 9.5–20 Hz) from representative segments of only 3 visually classified sleep stages (quiet waking, deep sleep and REM sleep), the sleep stage being the dependent variable. This results in 2 sleep stage-dependent canonical variables covering 100% of the variance in the data set and two residual canonical variables which are independent of sleep stage assignment. These 2 residual variables are subsequently used in

a second canonical discriminant analysis in which the presence or absence of the drug is used as the dependent variable, resulting in a single canonical variable (the drug score) associated with the drug effect on the sleep stage-independent variance of the EEG spectral parameters.

CRITICAL ASSESSMENT OF THE METHOD

According to the author's own judgment, antidepressants, antipsychotics and stimulants can be discriminated from each other and from placebo successfully from each other and from placebo by this method, whereas nootropics classified as placebo. Unfortunately, anxiolytics, hypnotics and anticonvulsants are classified poorly.

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E.5 Neuroleptic Activity

E.5.0.1 General Considerations

Neuroleptics have been defined as therapeutics effective against schizophrenia. One has to bear in mind that the effect of certain drugs has not been predicted by pharmacological tests but has been found in clinical trials by serendipity. The clinical discoveries were

followed by pharmacological studies in many laboratories (Courvoisier 1956).

Various studies have demonstrated the blockade of postsynaptic catecholamine receptors, especially D₂-receptors to be the main mode of action of most neuroleptics. Several *in vitro* methods measure the receptor blockade by neuroleptics.

Pharmacological models in the development of antipsychotic drugs were reviewed by Costall et al. (1991).

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E.5.1

In Vitro Methods

E.5.1.1

D₁ Receptor Assay: [³H]-SCH 23390 Binding to Rat Striatal Homogenates

PURPOSE AND RATIONALE

Dopamine receptors are the primary targets in the development of drugs for the treatment of schizophrenia, Parkinson's disease and Huntington's chorea (Seeman and Van Tol 1994).

Reviews on dopamine receptors and their subtypes were given by Baldessarini and Tarazi (1996, Missale et al. 1998), and by the NC-IUPHAR subcommittee on dopamine receptors (Schwartz et al. 1998).

Multiple dopamine receptors are known. Two groups are most studied, designated as D₁ and D₂. In the group of D₁-like dopamine receptors the subtypes D_{1A} and D₅/D_{1B} have been described. To D₂-like dopamine receptors belong the D_{2S}, the D_{2L}, the D₃, and the D₄ receptor (Sokoloff et al. 1990; Civelli et al. 1991; Grandy et al. 1991; Van Tol et al. 1991; Lévesque et al. 1992; Baldessarini et al. 1993; Ginrich and Caron 1993; Todd and O'Malley 1993; Waddington and Deveney 1996).

D₁ receptors are positively linked to adenylate cyclase and the D₂ receptor has been shown to be negatively linked to adenylate cyclase. For typical neuroleptic agents, like butyrophenones, a good correlation was found between D₂ receptor binding and clinically effective doses. Atypical neuroleptics, like cloza-

pine, were found to be potent inhibitors of D₁ and D₄ receptor binding, renewing interest in these receptor types. The compound SCH 23390 was found to be selective for the D₁ receptor.

PROCEDURE

Reagents

[N-Methyl-³H] Sch 23390 (Amersham Lab., specific activity 67–73 Ci/mmol). For IC₅₀ determinations, ³H-Sch 23390 is made up to a concentration of 10 nM and 50 µl is added to each tube. This yields a final concentration of 0.5 nM in the assay.

d-Butaclamol (Ayerst Laboratories). A 1 mM stock solution is made and diluted 1:20.

20 µl are added to 3 tubes for the determination of nonspecific binding.

For the test compounds a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assays ranges from 10⁻⁵ to 10⁻⁸ M.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, striata dissected and weighed. The striata are homogenized in 100 volumes of 0.05 M Tris buffer, pH 7.7, using a Tekmar homogenizer. The homogenate is centrifuged at 40,000 g for 20 min and the final pellet is resuspended in the original volume of 0.05 M Tris buffer, pH 7.7, containing physiological ions (NaCl 120 mM, KCl 5 mM, MgCl₂ 1 mM and CaCl₂ 2 mM).

Assay

50 µl 0.5 M Tris buffer, pH 7.7, containing physiological ions

380 µl H₂O

20 µl vehicle or butaclamol or appropriate concentration of test compound

50 µl ³H-SCH 23390

500 µl tissue suspension.

The tubes are incubated at 37°C for 30 min. The assay is stopped by rapid filtration through Whatman GF/B filters using a Brandell cell harvester. The filter strips are then washed 3 times with ice-cold 0.05 M Tris buffer, pH 7.7, and counted in 10 ml Liquiscint scintillation cocktail.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 1 µM d-butaclamol. IC₅₀ calculations are performed using log-probit analysis. The percent inhibition at each drug

concentration is the average of duplicate determinations.

MODIFICATIONS OF THE METHOD

Wamsley et al. (1992) recommended the radioactive form of a dopamine antagonist, [³H]SCH39166 as ligand for obtaining selective labeling of D₁ receptors.

Sugamori et al. (1998) characterized the compound NNC 01-0012 as a selective and potent D_{1C} receptor antagonist.

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E.5.1.2

D₂ Receptor Assay: [³H]-Spiroperidol Binding

PURPOSE AND RATIONALE

The neuroleptic compound haloperidol has been used as binding ligand to study the activity of other neuroleptics. The use of haloperidol has been superseded by spiroperidol. Dopamine receptor binding assays employing dopaminergic antagonists in mammalian striatal tissue, a dopamine-enriched area of the brain, have been shown to be predictive of *in vivo* dopamine receptor antagonism and antipsychotic activity. Significant correlations exist between neuroleptic binding affinities and their molar potencies in antagonism of apomorphine- or amphetamine-induced stereotypy,

apomorphine-induced emesis in dogs, and antipsychotic activity in man. Spiroperidol is considered to be an antagonist specific for D₂ receptors.

PROCEDURE

Tissue Preparation

Male Wistar rats are decapitated, their corpora striata removed, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 40,000 *g* for 15 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 40,000 *g*. The final pellet is then resuspended in Tris buffer containing physiological salts (120 mM NaCl, 5 mM KCl, 2 mM CaCl₂ and 1 mM MgCl₂) resulting in a concentration of 10 mg/ml.

Assay

The membrane preparations are incubated with ³H-spiroperidol (0.25 nM) and various concentrations of test drug at 37°C for 20 min in a K/Na phosphate buffer (50 mM, pH 7.2), followed by cooling in an ice bath for 45 min. To determine non-specific binding, samples containing 10 mM (+)-butaclamol are incubated under identical conditions without the test compound.

Bound ligand is separated by rapid filtration through Whatman GF/B glass fiber filters. The filters are washed three times with ice-cold buffer, dried, and shaken thoroughly with 3.5 ml scintillation fluid. Radioactivity is determined in a liquid scintillation counter. Specific binding is defined as the difference between total binding and the binding in the presence of 2.0 mM (+)-butaclamol.

EVALUATION

The following parameters are determined:

- total binding of ³H-spiroperidol
- non-specific binding: binding of samples containing 2 mM butaclamol
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of the control value.

IC₅₀ values are determined using at least 3–4 different concentrations of the test compound in triplicate. Results are presented as mean ± standard deviation.

Dissociation constants (*K*_d) are determined, using ³H-spiroperidol concentrations ranging between 0.1 and 1.0 nM. *K*_i values (inhibitory constants) are cal-

culated using the following equation:

$$K_i = \frac{IC_{50}}{1 + c/K_d}$$

c = ³H-spiroperidol concentrations used to determine IC₅₀.

Standard values: *K*_i of haloperidol = 6.0 ± 1.2 nM.

MODIFICATIONS OF THE METHOD

Two isoforms of the D₂ receptor were found by alternative splicing: the long (D_{2L}) and the short (D_{2S}) isoform (Dal Toso et al. 1988; Giros et al. 1989; Monsma et al. 1989; Itokawa et al. 1996).

Niznik et al. (1985) recommended [³H]-YM-09151–2, a benzamide neuroleptic, as selective ligand for dopamine D₂ receptors.

Hall et al. (1985) used [³H]-eticlopride, a substituted benzamide, selective for dopamine-D₂ receptors, for *in vitro* binding studies.

Radioactive ligands for the D₂ and the D₃ receptor were described by Seeman and Schaus (1991), Chumpradit et al. (1994), Booze and Wallace (1995), Gackenheim et al. (1995), Seeman and van Tol (1995), Van Vliet et al. (1996).

Vessotskie et al. (1997) characterized binding of [¹²⁵I]S(–)5-OH-PIPAT to dopamine D₂-like receptors.

Neve et al. (1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells (McConnell et al. 1991, 1992; Owicki and Parce 1992). In C₆ glioma cells and L fibroblasts expressing recombinant dopamine D₂ receptors, the dopamine D₂ receptor agonist, quinpirole, accelerated the rate of acidification of the medium dose-dependent up to 100 nM quinpirole. The response was inhibited by the D₂ antagonist spiperone. The D₂ receptor stimulated acidification was due to transport of protons by a Na⁺/H⁺ antiporter which was verified by the inhibition with amiloride or methylisobutyl-amiloride.

The isolated rabbit ear artery was recommended as a useful model to characterize dopamine D₂ agonists and antagonists (Hieble et al. 1985).

Human Recombinant Dopamine D_{2A} and D_{2B} Receptors

Hayes et al. (1992) described functionally distinct human recombinant subtypes of the dopamine D₂ receptor, D_{2A} and D_{2B}.

D_{2A} Receptor Binding

In a radioligand binding assay the binding of [³H]-spiperone to membranes prepared from COS cells

transiently expressing a recombinant human dopamine D_{2A} receptor is measured.

Twenty mg of membrane is incubated with [³H]-spiperone at a concentration of 2.0 nM for 2 h at 25°C. Non-specific binding is estimated in the presence of 10 mM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [³H]-spiperone bound.

D_{2B} Receptor Binding

In a radioligand binding assay the binding of [³H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{2B} receptor is measured.

Fifteen mg of membrane is incubated with [³H]-spiperone at a concentration of 0.7 nM for 2 h at 37°C. Non-specific binding is estimated in the presence of 10 mM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [³H]-spiperone bound.

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E.5.1.3

Dopamine D₂ Receptor Autoradiography (³H-Spiperone Binding)

PURPOSE AND RATIONALE

Autoradiography of ³H-spiperone binding sites using selective labeling conditions permits the visualization of the anatomical locations of D₂-dopamine receptors (Palacios et al. 1981). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Altar et al. 1984, 1985). Using autoradiographic techniques, it has been demonstrated that striatal D₂ receptors are present on intrinsic neurons (Trugman et al. 1986; Joyce and Marshall 1987) and that the distribution of D₂ receptors within the striatum is not homogeneous (Joyce et al. 1985). Anatomically discrete interactions of drugs with D₂ receptors can be examined *in vitro* with inhibition experiments and *ex vivo* following acute or chronic drug treatment of the whole animal.

Since ³H-spiperone labels serotonin-2 (5-HT₂) sites in many brain regions, a masking concentration of a 5-HT₂ receptor blocker, e. g., ketanserin, is included to selectively define binding to D₂ receptors. This is necessary if the test compound inhibits 5-HT₂ binding, or if the brain region of interest has a low D₂ receptor density.

The assay is used to determine potential antipsychotic activity of compounds via direct interaction with the D₂ dopamine recognition site in discrete regions of the rat brain.

PROCEDURE

Reagents

- 1a. 0.5 M Tris+1.54 M NaCl, pH 7.4
- 1b. 0.05 M Tris+0.154 M NaCl, pH 7.4
2. ³H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham (TRK.818).
For IC₅₀ determinations: ³H-spiperone is prepared at a concentration of 8 nM and 0.55 ml is added to each slide mailer (yields a final concentration of 0.4 nM in the 11.0 ml assay volume).

For saturation experiments: ³H-spiperone is prepared at a concentration of 20 nM. The final concentrations should range from 0.2–1.0 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).

3. Sulpiride is obtained from Sigma. A stock solution of 5×10^{-4} M is made by dissolving the sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 10 μM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).
4. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin in 0.5 ml 1 N acetic acid and bringing the final volume to 10 ml with distilled water. The tartrate salt is water-soluble. This is further diluted to 5×10^{-6} M (50 μl q.s. to 10 ml). 0.22 ml is added to all mailers.
5. Test compounds (for IC₅₀ determinations). For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Rat brain sections are collected from plates 9 (rostral nucleus accumbens) through plate 17 (caudal striatum) of "The Rat Brain Atlas in Stereotaxic Coordinates" by Paxinos and Watson.

1. For *in vitro* inhibition experiments, 3–5 sets of 10 slides are collected with 3–4 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 3–4 sections per slide.
3. For *ex vivo* inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, 2 sections per slide are collected.

Assay

1. Preparation of slide mailers (11.0 ml volume/slide mailer):

Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is

sufficient to cover 2 sections. A proportional adjustment of the volumes to be pipetted is made.

a) *In vitro* inhibition experiments

Separate mailers are prepared for total binding, nonspecific binding and 7–8 concentrations of test compound. Ketanserin is included in all mailers to mask binding of [³H]-spiperone to 5-HT₂ sites so that inhibition of binding is D₂-selective.

5.50 ml buffer 1b

0.55 ml buffer 1a

0.55 ml [³H]-spiperone, 0.4 nM final concentration

3.96 ml distilled water

0.22 ml ketanserin, 5 × 10⁻⁶ M, final concentration 100 nM or vehicle

0.22 ml test compound, final concentration 10⁻⁸–10⁻⁵ M or sulpiride 5 × 10⁻⁴, final conc. 10 μM or vehicle

b) *Ex vivo* inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including ketanserin to mask 5-HT₂ receptor binding.

c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Ketanserin is not included in the mailers, in saturation experiments, since specific binding is defined as sulpiride-displaceable.

5.50 ml buffer 1b

0.55 ml buffer 1a

0.55 ml [³H]-spiperone, final concentration 0.2–1.0 nM

4.18 ml distilled water

0.22 ml 5 × 10⁻⁴ M sulpiride, final concentration 10 μM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris+0.154 M NaCl, pH 7.4 for 5 min and further incubated for 60 min with [³H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, rinsed in buffer 1b for 2 × 5 min, and dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter and radioactivity is counted after addition of 10 ml of scintillation fluid. Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually over night). Slides are then

mounted onto boards, along with ³H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrafilm for 14–17 days.

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E.5.1.4

Binding to the D₃ Receptor

PURPOSE AND RATIONALE

Sokoloff et al. (1990) reported molecular cloning and characterization of a dopamine receptor (D₃) as a potential target for neuroleptics. The D₃ receptor is localized in limbic areas of the brain which are associated with cognitive, emotional and endocrine functions. Together with the D_{2S}, the D_{2L} and the D₄ receptor, the D₃ receptor belongs to the group of D₂-like dopamine receptors (Ginrich and Caron 1993). 7-[³H]hydroxy-*N,N*-di-*n*-propyl-2-aminotetralin (Lévesque et al. 1992), R(+)-7-OH-DPAT (Balldassarini et al. 1993; and [¹²⁵I]trans-7-OH-PIPAT-A (Kung et al. 1993) have been recommended as ligands for receptor binding studies.

Chio et al. (1993) compared the heterologously in Chinese hamster ovary cells expressed D₃ dopamine receptors with D₂ receptors.

Damsma et al. (1993) described R-(+)-7-OH-DPAT (R-(+)-7-hydroxy-2-(N,N-di-n-propylamino)tetralin) as a putative dopamine D₃ receptor ligand.

Functional correlates of dopamine D₃ receptor activation in the rat *in vivo* and their modulation by the selective agonist, (+)-S 14297, have been described by Millan et al. (1995).

Isoforms of the D₃ receptor have been described (Pagliusi et al. 1993).

Akunne et al. (1995) described binding of the selective dopamine D₃ receptor agonist ligand [³H]PD 128907=4aR,10bR-(+)-trans-3,4,4a,10b-tetrahydro-4-n-propyl-2H,5H-[1]benzopyrano[4,3-b]1,4-oxazin-9-ol.

PROCEDURE

Human dopamine D₃ receptor is expressed in Chinese hamster ovary cells. Cells are grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells are harvested by trypsin treatment (0.25%) for 4–5 min and centrifugation at 2000 g for 5 min. They are homogenized with a Polytron in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and are centrifuged at 35,000 g for 15 min. The pellet is then resuspended by sonication in a buffer containing 50 mM NaHepes, 1 mM EDTA, 50 μM 8-hydroxyquinoline, 0.005% ascorbic acid, and 0.1% bovine serum albumin (pH 7.5) (incubation buffer). Membrane suspensions (15–25 μg protein) are added to polypropylene test tubes containing [³H]7-OH-DPAT (7-[³H]hydroxy-N,N-di-n-propyl-2-aminotetralin) for the D₃ receptor assay. Competing drugs are dissolved in incubation buffer, the final volume being 1 ml. Tubes are incubated in triplicate for 1 h at room temperature. The incubations are stopped by rapid filtration under reduced pressure through Whatman GF/C glass filters coated with 0.1% bovine serum albumin, followed by 3 rinses with 3–4 ml ice-cold buffer. Non-specific binding is measured in the presence of 1 μM dopamine.

EVALUATION

Saturation curves are analyzed by computer non-linear regression using a one-site cooperative model to obtain equilibrium dissociation constants (K_D) and maximal density of receptors (B_{max}). Inhibition constants (K_i) are estimated according to the equation

$$K_i = IC_{50}/1 + L/K_D$$

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E.5.1.5

Binding to D₄ Receptors

PURPOSE AND RATIONALE

Van Tol et al. (1991) reported cloning of the gene of a human dopamine D₄ receptor with high affinity for the antipsychotic clozapine. Together with the D_{2S}, the D_{2L} and the D₃ receptor, the D₄ receptor belongs to

the group of D₂-like dopamine receptors (Ginrich and Caron 1993). Recognition and characterization of this dopamine binding site may be useful in the design of new types of antipsychotic drugs.

Dopamine D₄ receptors have been localized in GABAergic neurons of the primate brain (Mrzljak et al. 1996).

PROCEDURE

A plasmid construct of a 3.9-kb gene-cDNA hybrid subcloned into the expression vector pCD-PS is introduced into COS-7 cells by calcium phosphate mediated transfection. Cells are cultivated and homogenized (Teflon pestle) in 50 mM Tris-HCl (pH 7.4 at 4°C) buffer containing 5 mM EDTA, 1.5 mM CaCl₂, 5 mM KCl, and 120 mM NaCl. Homogenates are centrifuged for 15 min at 39,000 *g*, and the resulting pellets resuspended in buffer at a concentration of 150–250 µg/ml. For saturation experiments, 0.25 ml of tissue homogenate are incubated in duplicate with increasing concentrations of [³H]spiperone (70.3 Ci mmol⁻¹; 10–3000 pM final concentration) for 120 min at 22°C in a total volume of 1 ml. For competition binding experiments, assays are initiated by the addition of 0.25 ml membrane and incubated in duplicate with various concentrations of competing ligands (10⁻¹⁴–10⁻³ M) and [³H]spiperone (150–300 pM) either in the absence or the presence of 200 µM Gpp(NH)p for 120 min at 22°C. Assays are terminated by rapid filtration through a Titertek cell harvester and filters then monitored for tritium. For all experiments, specific binding is defined as that inhibited by 10 µM (-)sulpiride.

EVALUATION

Both saturation and competition binding data are analyzed by the non-linear least-square curve-fitting program LIGAND run on a suitable PC.

MODIFICATIONS OF THE METHOD

Human Recombinant Dopamine D_{4,2}, D_{4,4}, D_{4,7} and D₅ Receptors

Van Tol et al. (1992) described multiple dopamine D₄ receptor variants in the human population.

Sunahara et al. (1991) reported the cloning of the gene for a human D₅ receptor.

Human Recombinant Dopamine D_{4,2} Receptor Binding

In a radioligand binding assay the binding of [³H]-spiperone to membranes prepared from COS cells

transiently expressing a recombinant human dopamine D_{4,2} receptor is measured.

Fifteen µg of membrane is incubated with [³H]-spiperone at a concentration of 0.7 nM for 2 h at 25°C. Non-specific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [³H]-spiperone bound.

Human Recombinant Dopamine D_{4,4} Receptor Binding

In a radioligand binding assay the binding of [³H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{4,4} receptor is measured.

Twenty-five µg of membrane are incubated with [³H]-spiperone at a concentration of 1.0 nM for 2 h at 25°C. Nonspecific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [³H]-spiperone bound.

Human Recombinant Dopamine D_{4,7} Receptor Binding

In a radioligand binding assay the binding of [³H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{4,7} receptor is measured.

Fifteen µg of membrane is incubated with [³H]-spiperone at a concentration of 0.7 nM for 2 h at 25°C. Non-specific binding is estimated in the presence of 10 µM haloperidol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [³H]-spiperone bound.

Human Recombinant Dopamine D₅ Receptor

In a radioligand binding assay the binding of [³H]SCH 23390 to membranes prepared from COS cells expressing a recombinant human dopamine D₅ receptor is measured.

First, 40 µg of membrane is incubated with [³H]SCH 23390 at a concentration of 2 nM for 2 h at 25°C. Non-specific binding is estimated in the presence of 10 µM *cis*-flupentixol. Membranes are filtered and washed 3 times with binding buffer, and filters are counted to determine [³H]SCH 23390 bound.

Several selective dopamine D₄ antagonists were described: Hikada et al. (1996), Merchant et al. (1996), Rowley et al. (1996), Bristow et al. (1997).

Some radioligands were proposed as being selective for dopamine D₄ receptors: [³H]clozapine (Ricci et al. 1997a, b), [³H]NGD 94–1 (Thurkauf 1997; Primus et al. 1997), RBI-257 (Kula et al. 1997).

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E.5.1.6

Determination of Dopamine Autoreceptor Activity

PURPOSE AND RATIONALE

The method describes the procedure to determine if a compound possesses autoreceptor blocking activity without the interference from postsynaptic effects. Striatal DOPA (3,4-dihydroxyphenylalanine), DOPAC (3,4-dihydroxyphenylacetic acid) and DA (dopamine) are quantitated following *in vivo* treatment with drug, apomorphine, gamma butyrolactone and NSD-1015. Antipsychotic compounds that block striatal dopaminergic presynaptic autoreceptors are believed to possess a greater liability for producing EPS.

PROCEDURE

Reagents

- 0.1 M HCl
- 1 N NaOH
- 0.1 M perchloric acid (PCA) containing 4.3 mM EDTA
- 2 mM solutions of DOPAC, DA and DOPA in 0.1 M HCl, 0.5 ml aliquots are stored at –60°C until use.
- Preparation of 2° standard mixture
10 μM solution of DOPAC, DA and DOPA diluted from reagent 4 with 0.1 M PCA/EDTA;
The 2° standard solution is used for the preparation of standard curves.
- Mobile phase/MeOH-buffer (4:96, v/v) buffer: 0.012 mM sodium acetate, 0.036 M citric acid and 152 μM sodium octane sulfonate, mobile phase: methanol/buffer (80 ml + 1920 ml) is filtered through a 0.2 μm nylon 66 filter.
- Preparation of dosing solutions
 - Apomorphine (2 mg/kg) is prepared in saline containing 1% Tween 80 + 0.1% ascorbic acid to prevent oxidation.
 - GBL (750 mg/kg) is prepared as a solution in saline containing 1% Tween 80.
 - NSD-1015 (100 mg/kg) is prepared as a solution in saline containing 1% Tween 80.

HPLC-Instrumentation

Consists of a

- pump, model SP8810 (Spectra Physics),
- injector, WISP 710B (Waters Associates),
- detector, 5100A electrochemical with a 5011 analytical cell and 5020 guard cell (ESA),

- integrator, D-2000 (Hitachi), used as a back-up for the data collection/integrator, CS 9000 (IBM) system
- analytical column: C18-ODS Hypersil, 3 μm , 100 \times 4.6 mm (Shandon)

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation at the pre-determined time. The brain is rapidly removed, the striatum is dissected on ice and frozen on dry ice. The tissue is analyzed by HPLC the same day.

Tissue is homogenized in 500 μl 0.1 M PCA/EDTA. The homogenate is centrifuged for 6 min using a microcentrifuge (model 5413, Eppendorf). The supernatant is transferred to 0.2 μm microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

Five μl of the striatum homogenate is injected into the HPLC column.

HPLC flow rate is 1.5 ml/min, run time is 20 min. Helium flow is constant in mobile phase.

For protein analysis, 1.0 ml 1 N NaOH is added to the tissue pellet. The next day, the protein analysis is performed as described by Bradford (1976) using the BioRad assay kit.

EVALUATION

Peak area is used for quantitation. The mg of protein and pmoles of DOPAC, DA and DOPA are calculated from linear regression analyses using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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E.5.1.7

Dopamine-Sensitive Adenylate Cyclase in Rat Striatum

PURPOSE AND RATIONALE

Agonist stimulation of dopamine D₁ receptors leads to increased cAMP formation mediated by a guanine nucleotide-binding regulatory protein. This effect is blocked by selective antagonists like SCH 23390.

Agonist stimulation of the dopamine D₂ receptor leads to a decreased cAMP formation mediated by a guanine nucleotide binding protein. Apomorphine is a potent agonist with full intrinsic activity at D₂ receptors. Phenothiazines block both D₁ and D₂ receptors, whereas butyrophenones and related drugs are very potent antagonists at D₂ receptors.

Studies on cAMP formation may be useful for differentiation of antipsychotic drugs.

PROCEDURE

Tissue Preparation

Male Wistar rats are sacrificed by decapitation, the brains removed, and the striata dissected out and weighed. Striatal tissue from 2 rats is homogenized in 25 volumes of ice-cold 0.08 M Tris-maleate buffer, pH 7.4, containing 2 mM EGTA. Protein content of an aliquot is determined. A 50 μl aliquot is used in the cyclase enzyme assay.

Enzyme Assay

The following volumes are placed in conical centrifuge tubes kept in an ice-water bath:

200 μl incubation buffer (equal amounts of 0.8 mM Tris-maleate, pH 7.4; 60 mM MgSO₄; 100 mM theophylline and 4 mM EGTA)

50 μl 1 mM dopamine HCl or water

25 μl test drug or water

125 μl distilled water

50 μl tissue homogenate

After incubation for 20 min at 0°C, the enzyme reaction is started by addition of 50 μl of 15 mM ATP solution. The tube rack is placed in a shaking water bath preset at 30°C for 2.5 min. The reaction is terminated by placing the tube rack in a boiling water bath for 4 min. Then, the tubes are centrifuged at 1000 g for 10 min.

A 25 μl aliquot of the supernatant in each tube is removed and the cAMP determined using a commercial RIA kit (Amersham).

EVALUATION

Results are expressed as pmoles cAMP/mg protein of dopamine stimulated vs. non dopamine stimulated level. Percentage inhibition of this dopamine stimulated level by test drugs is calculated and IC_{50} values determined by log-probit analysis.

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E.5.1.8 **α_1 -Adrenergic Receptor Binding in Brain****PURPOSE AND RATIONALE**

The use of neuroleptic and antidepressant drugs is sometimes limited by their side effects, such as orthostatic hypotension and sedation. These side effects are attributed to blockade of central and peripheral adrenergic α -receptors. For neuroleptics the ratio between their dopamine antagonistic and their α -receptor antagonistic potencies should be taken into account rather than their absolute α -blocking effect. WB-4101 is a specific and potent antagonist of the

α_1 -adrenoreceptor, characterized *in vitro* in rat brain, heart, vascular smooth muscle and gastrointestinal smooth muscle.

The *in vitro* [³H]-WB 4101 receptor binding assay quantitates the α -adrenergic blocking properties of psychoactive agents and is used to assess a compound's potential to cause orthostatic hypotension and sedation as well as primary blood pressure lowering effects through α_1 -receptor blockade.

PROCEDURE**Reagents**

[Phenoxy-3-³H(N)]-WB 4101 = (2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane, New England Nuclear, (specific activity 20–35 Ci/mmol).

For IC_{50} determinations [³H]-WB 4101 is made up to a concentration of 2 nM in Tris buffer and 500 μ l is added to each tube (yields a final concentration of 0.5 nM in the 2 ml assay)

L-norepinephrine bitartrate (Sigma Chemical Company). A 800 μ M solution is prepared in Tris buffer and 250 μ l is added to each of 3 tubes to determine non-specific binding. This yields a final concentration of 100 μ M in the 2 ml assay.

Test compounds: A 80 μ M stock solution is made up in a suitable solvent and serially diluted with Tris buffer, such that the final concentration in the assay ranges from 10⁻⁵ to 10⁻⁸ M. Usually, seven concentrations are studied for each assay.

Tissue Preparation

Male Wistar rats (100–150 g) are sacrificed by decapitation. The whole brain minus cerebellum is homogenized in 75 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 40,000 g at 4°C for 15 min. The supernatant is discarded and the pellet is rehomogenized in fresh Tris buffer and recentrifuged at 40,000 g at 4°C for 15 min. The final pellet is resuspended in the original volume of ice-cold 0.05 M Tris buffer. The final tissue concentration in the assay is 10 mg/ml. Specific binding is approximately 80% of total bound ligand.

Assay

1200 μ l tissue suspension

500 μ l ³H-WB 4101

250 μ l vehicle (for total binding) or

800 μ M L-norepinephrine bitartrate (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept in ice for additions, then vortexed and incubated for 15 min at 25°C. The binding is terminated by rapid vacuum filtration through Whatman GF/B filters, followed by three 5 ml washes with ice-cold 0.05 M Tris buffer. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

Specific WB 4101 binding is defined as the difference between the total binding and that bound in the presence of 100 µM norepinephrine. IC_{50} calculations are performed using computer-derived log-probit analysis.

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E.5.1.9

[³H]Spiroperidol Binding to 5-HT₂ Receptors in Rat Cerebral Cortex

PURPOSE AND RATIONALE

The purpose of this assay is to determine the anti-serotonin activity of neuroleptics, antidepressants and antihypertensive compounds, by measuring the displacement of [³H]spiroperidol from serotonergic antagonist

binding sites in cerebral cortical membranes. The regulation of 5-HT₂ receptor density by chronic antidepressant treatment is discussed in a separate protocol (see Sect. E.6.1.6).

The receptor binding of serotonergic sites in the CNS has been investigated using [³H]serotonin (5-HT) (Bennett and Snyder 1976), [³H]LSD (Peroutka and Snyder 1979) and [³H]spiroperidol (Peroutka and Snyder 1979; List and Seeman 1981; Leysen et al. 1978) as the radioligand. Receptor sites have been defined kinetically and classified as 5-HT₁ sites (labeled by [³H]5-HT, displaced by agonists) and 5-HT₂ sites (labeled by [³H]-spiroperidol and displaced by antagonists). [³H]LSD labels both 5-HT₁ and 5-HT₂ binding sites (Peroutka and Snyder 1979). Of the brain regions tested, the frontal cerebral cortex contained the greatest density of 5-HT₂ binding sites. Lesioning studies indicate that 5-HT₂ binding sites are post synaptic and not linked to adenylate cyclase (Peroutka et al. 1979).

The inhibition of 5-HT₂ binding correlates with the inhibition of quipazine-induced head twitch, which may reflect decreased behavioral excitation. The physiological and pharmacological role of these receptors is not clear. Although numerous neuroleptics and antidepressants of varying chemical structures are potent inhibitors of 5-HT₂ binding, there is no clear-cut relationship to the efficacy of these drugs. Methysergide and cyproheptadine are both potent inhibitors of 5-HT₂ binding without having neuroleptic or antidepressant effects. However, potent interaction with 5-HT₂ receptors may indicate a reduced potential for catalepsy, since methysergide blocks catalepsy induced by haloperidol (Rastogi et al. 1981). The interaction of serotonergic neurons with cholinergic neurons in the striatum (Samanin et al. 1978) may also be decreased by potent 5-HT₂ antagonists. In addition, the ratio of activity at D₂ and 5-HT₂ receptors may be useful in the screening of atypical antipsychotic agents (Meltzer et al. 1989). Furthermore, it has been shown that ketanserin, a selective 5-HT₂ antagonist, is an effective hypotensive agent which blocks peripheral vascular 5-HT receptors.

5-HT₂ receptors have been subdivided into 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors. The new 5-HT receptor classification has been published by the VII. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin) (Hoyer et al. 1994). Further comments were given by Humphrey et al. (1993), Martin and Humphrey (1994), Saxena (1994), Tricklebank (1996).

Several compounds with HT_{2A} antagonistic activity are described, such as tradozone (Clements-Jewery

et al. 1980; Hingtgen et al. 1984; Stryjer et al. 2003), MDL 100,907 (Kehne et al. 1996; Moser et al. 1996), and sarpogrelate (Hayashi et al. 2003).

McCullough et al. (2006) described the 5-HT_{2B} antagonist and 5-HT₄ agonist activities of tegaserod in the anesthetized rat.

PROCEDURE

Reagents

- 0.5 M Tris buffer, pH 7.7
 - 57.2 g Tris HCl
16.2 g Tris base
q.s. to 1 liter (0.5 M Tris buffer, pH 7.7)
 - Make a 1:10 dilution in distilled H₂O (0.05 M Tris buffer, pH 7.7)
- Tris buffer containing physiological ions
 - Stock buffer
NaCl 7.014 g
KCl 0.372 g
CaCl₂ 0.222 g
MgCl₂ 0.204 g
q.s. to 100 ml in 0.5 M Tris buffer
 - Dilute 1:10 in distilled H₂O
This yields 0.05 M Tris HCl, pH 7.7; containing NaCl (120 mM), KCl (5 mM), CaCl₂ (2 mM) and MgCl₂ (1 mM)
- [Benzene-³H] spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear. For IC₅₀ determinations: ³H-spiroperidol is made up to a concentration of 30 nM in 0.01 N HCl and 50 μl added to each tube (yields a final concentration of 1.5 nM in the 1 ml assay).
- Methysergide maleate is obtained from Sandoz. Methysergide maleate stock solution is made up to 0.25 mM for determination of nonspecific binding. The final concentration in the assay is 5 μM, when 20 μl of the stock solution is added to the reaction tube.
- Test compounds. For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10⁻⁵ to 10⁻⁸ M. Seven concentrations are used for each assay and higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated and the cerebral cortical tissue is dissected, weighed and homogenized in 50 volumes of 0.05 M Tris buffer, pH 7.7 (buffer 1b) with the Brinkman Polytron, then centrifuged at

40,000 g for 15 min. The supernatant is discarded and the pellet resuspended and recentrifuged as described above. This pellet is resuspended in 50 volumes of buffer 2b and stored in an ice bath. The final tissue concentration is 10 mg/ml. Specific binding is 7% of the total added ligand and 50% of total bound ligand.

Assay

- | | |
|--------|-----------------------------------------------------------------------------------------------------------------|
| 50 μl | 0.5 M Tris-physiological salts (buffer 2a) |
| 380 μl | H ₂ O |
| 20 μl | vehicle (for total binding) or 0.25 mM methysergide (for nonspecific binding) or appropriate drug concentration |
| 50 μl | [³ H]spiroperidol |
| 500 μl | tissue suspension |

The samples are incubated for 10 min at 37°C, then immediately filtered under reduced pressure using Whatman GF/B filters. The filters are washed with three 5 ml volumes of ice-cold 0.05 M Tris buffer, pH 7.7 mM methysergide.

EVALUATION

IC₅₀ calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATION OF THE METHOD

The receptor binding properties of the 5-HT₂ antagonist ritanserin were reported by Leysen et al. (1985).

Preclinical characterization of a putative antipsychotic as a potent 5-HT_{2A} antagonist was reported by Kehne et al. (1996).

Using [¹²⁵I]LSD and [³H]5-HT binding assays Siegel et al. (1996) characterized a structural class of 5-HT₂ receptor ligands.

[³H]Ketanserin has been described as a selective ³H-ligand for 5-HT₂ receptor binding sites. (Leysen et al. 1981).

[³H]RP 62203, a potent and selective 5-HT₂ antagonist was recommended for *in vivo* labeling of 5-HT₂ receptors (Fajolles et al. 1992).

Other selective 5-HT₂ receptor radioligands were recommended:

[¹²⁵I]-EIL (radioiodinated D-(+)-N1-ethyl-2-iodo-lysergic acid diethylamide) (Lever et al. 1991); [³H]-MDL100,907 (Lopez-Gimenez et al. 1998).

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Serotonin 5-HT₂ Receptor Autoradiography (³H-Spiperone Binding)

PURPOSE AND RATIONALE

Autoradiography of ³H-spiperone binding sites with selective labeling conditions permits the visualization of the anatomical locations of 5-HT₂ receptors (Palacios et al. 1981; Pazos et al. 1985; Altar et al. (1985). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Pazos et al. 1985; Altar et al. (1984). Using autoradiographic techniques, it has been demonstrated that there is a heterogeneous distribution of 5-HT₂ receptors, with much higher levels in telencephalic areas such as the neocortex and the claustrum than in meso- or metencephalic areas. Within the cortex, 5-HT₂ receptors are abundant in layers IV and V (Pazos et al. 1985). The high concentration of 5-HT₂ receptors in the frontoparietal motor area and the claustrum which connects to the motor cortex and other motor areas suggests a physiological role for 5-HT₂ receptors in some motor syndromes (Cadet et al. 1987; Costall et al. 1975; Kostowski et al. 1972). The high affinity of the atypical antipsychotic clozapine for 5-HT₂ receptors (Fink et al. 1984; Altar et al. (1986) and the down-regulation of 5-HT₂ receptors following chronic administration of clozapine (Reynolds et al. 1983; Lee and Tang 1984; Wilmot and Szczepanik 1989) suggests that 5-HT₂ receptor interaction may be a significant factor in the lack of extrapyramidal side effects and tardive dyskinesias with its clinical use.

Since ³H-spiperone labels α_1 -noradrenergic sites in the cerebral cortex, a masking concentration of the α_1 -blocker prazosin is included to selectively define binding to 5-HT₂ receptors (Morgan et al. 1984). This is necessary if the test compound also inhibits α_1 -receptors which may be present in the brain region of interest.

The assay is used to determine the direct interaction of potential antipsychotic compounds with the

serotonin-5-HT₂ recognition site in discrete regions of the rat brain either *in vitro* or after *ex vivo* treatment of the whole animal.

PROCEDURE

Reagents

- 1a. 0.5 M Tris+1.54 M NaCl, pH 7.4
- 1b. 0.05 M Tris+0.154 M NaCl, pH 7.4
2. ³H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham.
 - For IC₅₀ determinations: ³H-spiperone is made up to a concentration of 20 nM and 0.55 ml is added to each slide mailer (yields a final concentration of 1.0 nM in the 11.0 ml assay volume).
 - For saturation experiments: ³H-spiperone is made up to a concentration of 20 nM. The final concentration should range from 0.5–2.5 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).
3. Methysergide is used to determine nonspecific binding in brain sections of the frontal cortex. Methysergide maleate is obtained from Sandoz. A stock solution of 2.5×10^{-4} M is made by dissolving in distilled water. A volume of 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 5 μ M). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).
4. Ketanserin is used to determine nonspecific binding in those slide mailers containing sections with the nucleus accumbens and striatum. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin (free base) in 0.05 N acetic acid or the tartrate salt in distilled water. This is further diluted to 5×10^{-6} M (50 μ M q.s 10 ml with distilled water). A volume of 0.22 ml is added to the slide mailers to give a final concentration of 100 nM.
5. Prazosin is used to mask α_1 -receptors in cortical brain section. Prazosin HCl is obtained from Pfizer. A stock solution of 10^{-4} M is made by dissolving prazosin in 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. This is further diluted to 5×10^{-6} M (100 μ M q.s 10 ml). A volume of 0.22 ml is added to those slide mailers to

be used for cortical brain sections to give a final concentration of 100 nM.

- Sulpiride is used to mask D₂ receptor binding in brain sections from the nucleus accumbens and striatum.

Sulpiride is obtained from Sigma. A stock solution of 10⁻⁴ M is made by dissolving sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. A volume of 0.22 ml is added to the appropriate slide mailers to give a final concentration of 10 μM.

- Test compounds (for *in vitro* IC₅₀ determinations). For most assays, a 5 × 10⁻³ M stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10⁻⁵ to 10⁻⁸ M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Frontal cortical brain sections are collected from plates 5 through 8 and nucleus accumbens/striatal sections are collected from plates 9 (rostral n. accumbens) through plate 17 (caudal striatum) of "The Rat Brain Atlas in Stereotaxic Coordinates" by Paxinos and Watson.

- For *in vitro* inhibition experiments, 3–5 sets of 10 slides are collected with 4–5 sections per slide.
- For saturation experiments, 3–5 sets of 12 slides are collected with 4–5 sections per slide.
- For *ex vivo* inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
- For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, two sections per slide are collected.

Assay

- Preparation of slide mailers (11.0 ml volume/slide mailer):

Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover 2 sections. A proportional adjustment of the volumes to be pipetted is made.

a) *In vitro* inhibition experiments

Separate mailers are prepared for total binding, nonspecific binding and 7–8 concentrations of test compound.

- For frontal cortical brain sections, prazosin is included in all mailers to mask the binding of [³H]-spiperone to α₁-receptors and non-

specific binding is defined with 5 μM methysergide.

- 5.50 ml buffer 1b
- 0.55 ml buffer 1a
- 0.55 ml [³H]-spiperone, 1.0 nM final concentration
- 3.96 ml distilled water
- 0.22 ml prazosin 5 × 10⁻⁶ M, final concentration 100 nM or vehicle
- 0.22 ml test compound, final concentration 10⁻⁸–10⁻⁵ M or methysergide 2.5 × 10⁻⁴ M, final concentration 5 μM or vehicle

- For brain sections with the nucleus accumbens and striatum in which there is negligible binding of [³H]-spiperone to α₁-receptors, prazosin is not included. Since levels of 5-HT₂ receptors in these brain areas are low, 10 μM sulpiride is included in all mailers to mask the binding of [³H]-spiperone to D₂ receptors.

Ketanserin, final concentration of 100 nM, is used to determine nonspecific binding since methysergide has a weak affinity for D₂ receptors (IC₅₀ approximately 1–5 μM).

- 5.50 ml buffer 1b
- 0.55 ml buffer 1a
- 0.55 ml [³H]-spiperone, 1.0 nM final concentration
- 3.96 ml distilled water
- 0.22 ml sulpiride 5 × 10⁻⁴ M, final concentration 10 μM or vehicle
- 0.22 ml test compound, final concentration 10⁻⁸–10⁻⁵ M or ketanserin 5 × 10⁻⁵ M final concentration 100 nM or vehicle

b) *Ex vivo* inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including sulpiride to mask D₂ receptor binding with brain sections through the nucleus accumbens and striatum and prazosin to mask α₁-receptors in cortical brain sections.

c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Prazosin is not included in the mailers in saturation experiments, since specific binding is defined by methysergide which has negligible affinity for α₁-receptors.

- 5.50 ml buffer 1b

- 0.55 ml buffer 1a
- 0.55 ml [³H]-spiperone, final concentrations 0.5–2.5 nM
- 4.18 ml distilled water
- 0.22 ml 2.5 × 10⁻⁴ M methysergide, final concentration 5 μM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris+0.154 M NaCl, pH 7.4 for 5 min and further incubated for 60 min with [³H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, 2 × 5 min rinsed in buffer 1b, dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter and radioactivity is counted after addition of 10 ml of scintillation fluid. Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually overnight). Slides are then mounted onto boards, along with ³H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrafilm for 14–17 days.

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Binding to the Sigma Receptor

PURPOSE AND RATIONALE

Sigma receptors, as a class of binding sites in the brain, were originally described as a subtype of the opiate receptors. Efforts to develop less addicting opiate analgesics led to the study of several benzomorphan derivatives which produce analgesia without causing the classical morphine-induced euphoria. Unfortunately, these compounds, like N-allylnormetazocine (SKF 10,047) produced a variety of psychotic symptoms. This psychotomimetic effect is thought to be mediated by sigma receptors. This binding site is sensitive to many neuroleptics, most notably the typical antipsychotic haloperidol, leading to the hypothesis that drug interactions with the sigma site may be a new approach for the discovery of novel antipsychotics which are not dopamine receptor antagonists. D₂ receptor antagonism is thought to be linked with the occurrence of extrapyramidal symptoms in the form of hyperkinesia and Parkinson symptoms or tardive dyskinesia limiting the therapeutic use of traditional antipsychotic medication. It is hoped that ligands to the sigma receptor do not produce these adverse reactions. The sigma site is believed to be distinct from the binding site for the psychotomimetic drug phencyclidine.

PROCEDURE

Reagents

(+)-SKF 10,047 is prepared as a stock solution of 5 × 10⁻³ M with distilled water. 130 μl added to the 6.5 ml assay yields a final concentration of 10⁻⁴ M.

³H-(+)-SKF 10,047 (specific activity 40 Ci/mmol) is obtained from New England Nuclear. A 200 nM stock solution is made up with distilled water for IC₅₀ determinations. 325 μl added to each tube yields a final concentration of 10 nM in the 6.5 ml assay.

Test Compounds

A 5 mM stock solution is prepared in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

The assay utilizes slide-mounted cross-sections of brain tissue from male Hartley guinea pigs. Whole brain sections of 10 μ m thickness are obtained from the hippocampus, thaw-mounted onto gel-chrome alum subbed slides, freeze-dried and stored at -70°C until use. On the day of the assay, the sections are thawed briefly at room temperature until the slides are dry and then used in the assay at a final volume of 6.5 ml.

Assay

Incubation solutions are prepared in plastic slide mailer containers as follows:

3.250 ml 0.05 M Tris buffer, pH 7.7

2.470 ml distilled water

0.325 ml 0.5 M Tris buffer, pH 7.7

0.130 ml (+)-SKF 10,047 or vehicle

0.325 ml [^3H](+)-SKF 10,047

Dried slides with tissue sections are added to the slide mailers and incubated at room temperature for 90 min. Non-bound radioligand is removed by rinsing the slides sequentially in two 5-min rinses in ice-cold 0.05 M Tris buffer and a dip in ice-cold distilled water. The sections are either swabbed with Whatman GF/B filters for scintillation counting of tissue-bound radioligand or exposed to tritium-sensitive film for autoradiography of the binding sites.

EVALUATION

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M (+)-SKF 10,047 and is typically 60–70% of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

MODIFICATIONS OF THE METHOD

[^3H](+)-pentazocine has been recommended as a highly potent and selective radioligand for μ receptors (de Costa et al. 1989; DeHaven-Hudkins et al. 1992).

Classification of sigma binding sites into σ_1 and σ_2 receptors has been proposed (Walker et al. 1990; Quirion et al. 1992; Abou-Gharbia et al. 1993).

Hashimoto and London (1993) characterized [^3H]ifenprodil binding to σ_2 receptors in rat brain.

Ganapathy et al. (1999) provided evidence for the expression of the type 1 sigma receptor in the Jurkat human T lymphocyte cell line.

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- 10 μ M solution of NE, DOPAC, DA, HVA, 5HIAA and 5-HT (diluted from reagent 3) in mobile phase (reagent 5)
 - the 2° Standard solution is used for the preparation of standard curves.
5. Mobile phase/MeOH: buffer (7.5:92.5, v/v)
- buffer: 0.07 M sodium acetate, 0.04 M citric acid, 130 μ M EDTA and 230 μ M sodium octane sulfonate
 - mobile phase: methanol/buffer (150 ml + 1850 ml) is filtered through a 0.2 μ m nylon 66 filter

HPLC-Instrumentation

- pump, model SP8810 (Spectra Physics),
- injector, WISP 710B (Waters Associates),
- detector, 5100A electrochemical with a 5011 analytical cell and 5020 guard cell (ESA),
- integrator, D-2000 (Hitachi), used as a back-up for the data collection/integrator, CS 9000 (IBM) system,
- analytical column: C18-ODS Hypersil, 3 μ m, 100 \times 4.6 mm (Shandon)

Animal Treatment

Six rats per group (150–250 g) are dosed with 4–5 different concentrations of the putative antipsychotic drug; usual concentrations range from 0.03 to 30 mg/kg. At a predetermined time, usually 60 min, the rats are sacrificed.

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation. The brain is rapidly removed and placed on ice. The striatum, nucleus accumbens and/or prefrontal cortex are dissected and placed in 1.5 ml microcentrifuge tubes. The tubes are capped and immediately placed in dry ice. The frozen brain sections are stored at –60°C until HPLC analysis.

Tissue is homogenized in mobile phase (striatum: in 600 μ l, nucleus accumbens and prefrontal cortex: in 300 μ l). The homogenates are centrifuged for 6 min using a microcentrifuge (model 5413, Eppendorf). The supernatants are transferred to 0.2 μ m microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

The following volumes are injected to the HPLC column:

- striatum: 5 μ l; nucleus accumbens: 20 μ l; prefrontal cortex: 50 μ l.

E.5.1.12

Simultaneous Determination of Norepinephrine, Dopamine, DOPAC, HVA, HIAA, and 5-HT from Rat Brain Areas

PURPOSE AND RATIONALE

To measure the effects of potential antipsychotic drugs on catecholamines and indols, a quantitative method for the determination of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindolacetic acid (5HIAA) and 5-hydroxytryptamine (5-HT) from rat brain regions is used. These catecholamines and indols are measured in rat brain prefrontal cortex, nucleus accumbens and striatum.

PROCEDURE

Reagents

1. 0.1 M HCl
2. 1 N NaOH
3. 2 mM solutions of NE, DOPAC, DA, HVA, 5HIAA and 5-HT in 0.1 M HCl;
 - 0.5 ml aliquots are stored at –60°C until use.
4. Preparation of 2° Standard Mixture

- HPLC flow rate is 1.0 ml/min, run time is 25 min. Helium flow is constant in mobile phase.

For protein analysis, 1 N NaOH is added to the tissue pellets as follows:

- striatum: 1.0 ml;
- nucleus accumbens and prefrontal cortex: 0.5 ml.

The next day, the protein analysis is run in duplicate with 5 µl of striatum. 20 µl of nucleus accumbens and prefrontal cortex as described by Bradford (1976) using the BioRad assay kit.

EVALUATION

Peak area is used for quantitation. The mg of protein and pmoles of NE, DOPAC, DA, HVA, 5HIAA and 5-HT are calculated from linear regression analysis using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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E.5.1.13

Measurement of Neurotransmitters by Intracranial Microdialysis

PURPOSE AND RATIONALE

Methods to measure neurotransmitters and their metabolites in specific areas of the brain by microdialysis were introduced by Ungerstedt and his group (Ungerstedt et al. 1982; Zetterström et al. 1982, 1983; Zetterström and Ungerstedt 1983; Ungerstedt 1984; Stähle et al. 1991; Lindefors et al. 1989; Amberg and Lindefors (1989) and by Imperato and di Chiara 1984, 1985). In brain dialysis, a fine capillary fibre is implanted in a selected brain area. Low molecular weight compounds diffuse down their concentration gradients from the brain extracellular fluid into a physiological salt solution that flows through the capillary fibre at a constant rate. The fluid is collected and analyzed.

PROCEDURE

Several designs of dialysis probes have been used (Santiago and Westerink 1990; Kendrick 1991):

1. Horizontal Probe

A straight tube (Vita Fiber, 3 × 50 Amicon) with an outer diameter of 0.34 mm and a molecular weight cut-off of 50000 is used. The outer surface of the tube is porous and can easily be sealed by epoxy which is applied by passing the tube through a droplet of epoxy and then through a narrow hole corresponding to the outer diameter of the tube. The wall of the tube is sealed in this way except for the area where the dialysis is intended to take place. The length of this region can be varied from 2 to 8 mm depending upon which structure of the brain will be perfused. During the coating and all other handling of the tube it is supported by a thin tungsten or steel wire inserted into its lumen. One end of the tube is glued into a steel cannula (6 mm long, outer diameter 0.64 mm).

Male Sprague Dawley rats weighing 250–300 g are anesthetized with halothane and held in a stereotactic instrument. The animals are maintained under halothane anesthesia during the entire experiment.

Holes are drilled bilaterally (5.7 mm below and 1.5 mm in front of bregma) in the temporal bones after the temporal muscles have been retracted from the bones and folded away.

During the implantation, the cannula is held by the micromanipulator of the stereotactic instrument and the dialysis tube is passed horizontally through the brain through the holes drilled on both sides of the skull. A polyethylene tubing carrying the perfusion

fluid is connected to the steel cannula. The perfusate is collected at the other end.

2. Loop Probe

The probe is made of a flexible cellulosic tubing (Dow 50, outer diameter 0.25 mm). Both ends of the tube are inserted into 0.64 mm diameter steel tubes, one of which is bent in an angle. A very thin microsuture (0.1 mm in diameter) is inserted into the tube and positioned half between the steel tubes. Before implantation, the tube is moistened and bent in such a way that the two steel tubes are held closely together in the micromanipulator of the stereotactic instrument. A tungsten wire is inserted into the straight steel tube and passed down the lumen of the dialysis tube in order to stretch it and make it rigid enough to be implanted into the brain. The tube is implanted vertically and the steel cannulae are attached to the skull by dental cement. The tungsten wire is removed before starting the experiment. The cellulosic tube is flexible enough to withstand the bending at the lower end. The microsuture keeps the bend open.

Loop-shaped or U-shaped microdialysis probes have been used by several authors, e. g., Ichikawa and Meltzer (1990), Jordan et al. (1994), Westerink and Tuinte (1986), Auerbach et al. (1995).

3. Vertical Probe

The probe is sealed at one end by epoxy. The other end is glued into a 0.64-mm diameter steel tube. A thin inner cannula made of a steel tube or a glass capillary carries the fluid to the bottom of the dialysis tube where it leaves the inner capillary and flows upwards and leaves the probe by a lateral tube. This vertical probe can also be coated with epoxy. It is especially suited for reaching ventral parts of the brain and performing dialysis in small nuclei of the brain.

A similar device has been described for continuous plasma sampling in freely moving rats by Chen and Steger (1993).

Most of the commercially available microdialysis probes are based on this principle.

4. Commercially Available Microdialysis Probes

The microdialysis probes CMA/10, manufactured by Carnegie Medicine, Stockholm, Sweden, consist of a tubular membrane (polycarbonate; length: 3 mm; outside diameter: 0.50 mm; and inside diameter: 0.44 mm) glued to a cannula (outside diameter: 0.60 mm), and sealed with a glue at the tip (Stähler et al. 1991). The perfusion medium is carried to the dialyzing part of the probe by a thin cannula inside the

probe. The medium leaves the inner cannula through two holes, flows back between the membrane and the inner cannula, and is collected at the outlet of the probe. The perfusion medium is delivered by means of a high precision micro-syringe pump.

This probe was used by several authors, e. g., Wood et al. (1988), Benveniste et al. (1989), Rollema et al. (1989), Scheller and Kolb (1991), Wang et al. (1993), Kreiss and Lucki (1995), Fink-Jensen et al. (1996).

CMA/11 probes were used by Boschi et al. (1995), Romero et al. (1996), Gobert et al. (1997).

Dialysis fibres with a semipermeable membrane AN 69-HF, Hospal-Dasco, Bologna, Italy, were used by de Boer et al. (1994), Rayevsky et al. (1995), Arborelius et al. (1996), Gainetdinov et al. (1996), Tanda et al. (1996).

EVALUATION

Samples of the dialysate are collected for different time intervals and analyzed for neurotransmitters. For the **evaluation of neuroleptics**, most authors measured dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) by HPLC using appropriate detectors. See and Lynch (1996) analyzed dialysis samples for glutamate and GABA concentrations.

For the **evaluation of antidepressants** the concentrations of 5-hydroxytryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), or noradrenaline (NA) were measured in the effluent by HPLC. Wood et al. (1988), Egan et al. (1996) used 3-methoxytyramine accumulation as an index of dopamine release.

CRITICAL ASSESSMENT OF THE METHOD

The results obtained from brain dialysis depend on at least three variables: type of probe, post-implantation interval, and whether anesthetized or freely moving animals are used (Di Chiara 1990).

Several authors analyzed the diffusion processes underlying the microdialysis technique and described the limitations of the experiments (Jacobson et al. 1985; Amberg and Lindfors 1989; Benveniste et al. 1989; Scheller and Kolb 1991; Le Quellec et al. 1995).

As a matter of fact, brain microdialysis has been used for the evaluation of many drugs in various indications, such as:

- for **neuroleptics** by Ichikawa and Meltzer 1990; Meil and See 1994; Hernandez and Hoebel 1994; See et al. 1995; Schmidt and Fadaye 1995; Semba et al. 1995; Rayevsky et al. 1995; Fink-Jensen et al.

1996; See and Lynch 1996; Gainetdinov et al. 1996; Egan et al. 1996; Klitenick et al. 1996,

- for **antidepressants** by de Boer et al. 1994; Jordan et al. 1994; Arborelius et al. 1995; Ascher et al. 1995; Auerbach et al. 1995; de Boer 1995, 1996; Casanovas and Artigas 1996; Gobert et al. 1995; Ichikawa and Meltzer 1995; Kreiss and Lucki 1996; Petty et al. 1996; Potter 1996; Romero et al. 1995; Sharp et al. 1996; Tanda et al. 1996a, b,
- for studies in **Parkinson** models by Rollema et al. 1989; Parsons et al. 1991.

MODIFICATIONS OF THE METHOD

Ferrara et al. (1990) continuously monitored ethanol levels in the brain by microdialysis.

Hernandez and Hoebel (1994) performed simultaneous cortical, accumbens, and striatal microdialysis in freely moving rats.

Hegarty and Vogel (1995) assayed dopamine, DOPAC and HVA in the brain of rats after acute and chronic diazepam treatment and immobilization stress.

Casanovas and Artigas (1996) implanted microdialysis probes simultaneously in six different brain areas of rats (frontal cortex, dorsal striatum, ventral hippocampus, dorsal hippocampus, dorsal raphe nucleus, median raphe nucleus).

Benevise et al. (1984) determined extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis.

Boschi et al. (1995) showed that microdialysis of small brain areas in mice is feasible using the smallest commercially available probes.

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E.5.1.14

Use of Push-Pull Cannulae to Determine the Release of Endogenous Neurotransmitters

PURPOSE AND RATIONALE

Originally reported by Gaddum (1961), the push-pull cannula has become recognized and utilized as a powerful tool in conjunction with sufficiently sensitive assays to measure low levels of neuroregulator release in distinct brain areas *in vivo*. (Philippu 1984).

This method has been used for various purposes, e. g.,

- to perfuse the ventricles of the brain with drugs, or to determine the release of labelled or endogenous compounds in the CSF (Bhattacharya and Feldberg 1958; Korf et al. 1976),
- to perfuse distinct brain areas with drugs and to study their effects on functions of the central nervous system (Myers et al. 1976; Bhargava et al. 1978; Ruwe and Myers 1978),
- to inject labelled monoamines or amino acids and to investigate the resting or induced release of radioactive compounds and their metabolites (Sulser et al. 1969; Strada and Sulser 1971; Kondo and Iwatsubo 1978),
- to perfuse distinct brain areas with labelled transmitter precursors and to determine the patterns of release of the newly synthesized transmitters (Philippu et al. 1974; Chéramy et al. 1977; Nieoullon et al. 1977; Gauchy et al. 1980),
- to perfuse distinct brain areas of anesthetized and conscious animals and to determine the release of endogenous neurotransmitters in the perfusate (Dluzen and Ramirez 1991).

PROCEDURE

The superfusion of the hypothalamus of the conscious, freely moving rabbit has been described by Philippu et al. (1981), Philippu (1984). Rabbits of both sexes are anesthetized with 40 mg/kg sodium pentobarbital i.p. Guide cannulae are mounted on a metal plate which is

fixed on the skull with screws and dental cement. Some days after the operation, the guide cannulae are replaced with push-pull cannulae which are 4 mm longer than the guide cannulae, thus reaching the areas which are intended for superfusion. The push-pull cannulae are connected by tubing to two peristaltic pumps: one to push and another one to pull the fluid. The second pump is essential, because the superfusate is not directly collected from the side branch of the push-pull cannula, but from tubing which is connected to the side branch. The superfusate is automatically collected every 10 s in fraction collectors.

EVALUATION

The concentrations of neurotransmitters, e.g., epinephrine, norepinephrine, or dopamine are determined with appropriate analytical methods (Wolfensberger 1984) before and after stimulation.

MODIFICATIONS OF THE METHOD

Experiments in cats were described by Dietl et al. (1981), in rats by Tuomisto et al. (1983).

The cortical cup technique for collection of neurotransmitters has been described by Moroni and Pepeu (1984).

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function is to produce a long-term effect on the recipient neuron.

Acute administration of antipsychotics induces c-fos expression in several areas of the rat forebrain as was shown with immunocytochemical methods (Dragunow et al. 1990; Nguyen et al. 1992; Robertson and Fibiger 1992; MacGibbon et al. 1994). Fos protein is believed to act as an initiator of long-term cellular changes (neural plasticity) in response to a variety of extracellular stimuli, including drugs (Graybiel et al. 1990; Rogue and Vicendon 1992). Typical (e.g. haloperidol) and atypical (e.g. clozapine) neuroleptic drugs have different antipsychotic effects and side-effects. A differential FOS-protein induction in rat forebrain regions after haloperidol and clozapine treatment was found (Deutch et al. 1992; Fibiger 1994; Fink-Jensen and Kristensen 1994; Merchant et al. 1994; Sebens et al. 1995). The induction pattern of Fos-like immunoreactivity in the forebrain could serve as predictor of atypical antipsychotic drug activity (Robertson et al. 1994).

PROCEDURE

Groups of 4–6 male Wistar rats weighing 350–450 g are injected subcutaneously with saline (control) or with various doses of the standard drugs or compounds with putative antipsychotic activity. After two hours, the animals are deeply anesthetized by intraperitoneal injection of 100 mg/kg pentobarbital and perfused with 200 ml saline followed by 200 ml of 4% paraformaldehyde in phosphate buffer solution (PBS). Each brain is removed immediately after perfusion and placed in fresh fixative for at least 12 h.

After the postfixative period, 30- μ m sections are cut from each brain using a vibratome. Several antisera to detect Fos can be used, such as a sheep polyclonal anti-body directed against residues 2 to 16 of the N-terminal region of the Fos molecule, or a polyclonal antiserum raised in rabbits against Fos peptide (4–17 amino acids of human Fos).

Sections are washed 3 times with 0.02 mM PBS and then incubated in PBS containing 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections are then washed 3 times in PBS and incubated in PBS containing 0.3% Triton X-100, 0.02% azide and Fos primary antisera (diluted 1:200) for 48 h. The sections are then washed 3 times with PBS and incubated with a biotinylated rabbit antisheep secondary antibody (diluted 1:200) for 1 h. The sections are washed three times with PBS and incubated for 1 h with PBS containing 0.3% Triton X-100 and 0.5% avidin-biotinylated horseradish peroxidase com-

E.5.1.15

Fos Protein Expression in Brain

PURPOSE AND RATIONALE

The proto-oncogene c-fos encodes a 55,000 mol wt, 380 amino-acid phosphoprotein (FOS), which after translation in the cytoplasm, re-enters the nucleus and binds to DNA (Morgan and Curran 1989). C-fos induction can occur as a consequence of synaptic activation. An increase in fos immunoreactivity is associated with an increased metabolic demand on a neuron, i. e., a marker for neurons that are metabolically activated. Intermediate early genes such as c-fos have been tentatively classified or linked to third messengers, whose

plex. After 3 washes in PBS the sections are rinsed in 0.1 M acetate buffer, pH 6.0. Fos immunoreactivity is revealed by placing the sections in a solution containing 0.05% 3,3'-diaminobenzidine, 0.2% ammonium nickel sulfate and 0.01% H₂O₂. The reaction is terminated with a washing in acetate buffer. The sections are mounted on chrome-alum-coated slides, dehydrated and prepared for microscopic observation.

Drug induced changes in Fos-like immunoreactivity are quantified by counting the number of immunoreactive nuclei in the medial prefrontal cortex, nucleus accumbens, medial and dorsolateral striatum and the lateral septal nucleus. The number of Fos-positive nuclei are counted with a 550 × 550 μm grid placed over each of these regions with a 100 × magnification.

Typical and atypical antipsychotics can be classified on the basis of difference between Fos-like immunoreactivity in the nucleus accumbens and lateral striatum. For this purpose, the data are corrected for the effects which are produced by the injection procedure itself. The injection corrected value for the dorsolateral striatum is subtracted from the corresponding accumbal value for each drug dose.

This manipulation yields a value termed the atypical index, i. e. number of Fos-positive neurons in the nucleus accumbens minus the number in the lateral striatum = atypical index. A negative index indicates the probability of side-effects, like extrapyramidal syndrome, exerted by the typical neuroleptics, a positive value to be devoid of it.

EVALUATION

A one-way analysis of variance is performed on the cell count data for each dose and the corresponding vehicle control. If the analysis of variance is significant, multiple comparisons are performed by using the Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Graybiel et al. (1990) reported a drug-specific activation of c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum by amphetamine and cocaine.

Deutch et al. (1991) found that stress selectively increases Fos protein in dopamine neurons innervating the prefrontal cortex.

Gogusev et al. (1993) described modulation of C-fos and other proto-oncogene expression by phorbol diester in a human histiocytosis DEL cell.

Deutch et al. (1995) studied the induction of Fos protein in the thalamic paraventricular nucleus as locus of antipsychotic drug action.

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E.5.1.16

Neurotensin

E.5.1.16.1

General Considerations on Neurotensin and Neurotensin Receptors

Neurotensin is a 13-amino-acid peptide originally isolated from calf hypothalamus (Carraway and Leeman 1973). It is secreted by peripheral and neuronal tissues and produces numerous pharmacological effects in animals suggesting **analgesic** (Coguerel et al. 1988; Clineschmidt and McGuffin 1977; Smith et al. 1997), **wound healing** (Brun et al. 2005), **cardiovascular** (Carraway and Leeman 1973; Schaeffer et al. 1998; Seagard et al. 2000), **endocrine** (Rostene and Alexander 1997), **hypothermic** (Bissette et al. 1976; Benmoussa et al. 1996; Tyler-McMahon et al. 2000), and **antipsychotic** (Nemeroff 1986; Sarhan et al. 1997; Feifel et al. 1999; Kinkead et al. 1999; Cusack et al. 2000) actions. Neurotensin is even considered to be an endogenous neuroleptic (Ervin and Nemeroff 1988; Gully et al. 1995). Radke et al. (1998) studied synthesis and efflux of neurotensin in different brain areas after acute and chronic administration of typical and atypical antipsychotic drugs.

Neurotensin affects **gastrointestinal functions**, such as stimulating the growth of various gastrointestinal tissues (Feurle et al. 1987), modulating pre- and post-prandial intestinal motility (Pellissier et al. 1996), inhibiting gastric acid secretion (Zhang et al. 1989a), stimulating responses in rat stomach strips (Quirion et al. 1980), inducing contractile responses in intestinal smooth muscle (Unno et al. 1999), and maintaining gastric mucosal blood flow during cold water restraint (Zhang et al. 1989b; Xing et al. 1998).

Neurotensin acts as a **growth factor** on a variety of normal and cancer cells (Wang et al. 2000).

Like other neuropeptides, neurotensin is synthesized as part of a larger precursor which also contains neuromedin N, a six amino acid neurotensin-

like peptide belonging to the gastrin-releasing peptide/bombesin family (see J.3.1.8).

Several peptidic and non-peptidic neurotensin agonists and antagonists have been synthesized and analyzed in pharmacological tests as potential drugs mainly in psychopharmacology (Gully et al. 1995, 1996, 1997; Azzi et al. 1996; Castagliuolo et al. 1996; Chapman and See 1996; Mule et al. 1996; Hong et al. 1997; Johnson et al. 1997; Sarhan et al. 1997; Betancur et al. 1998; Gudasheva et al. 1998; Schaeffer et al. 1998; Kitabgi 2002). Furthermore, inhibitors of neurotensin-degrading enzymes were described (Bourdel et al. 1996). Binder et al. (2001) reviewed neurotensin and dopamine interactions.

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E.5.1.16.2

Neurotensin Receptor Binding

PURPOSE AND RATIONALE

Neurotensin interacts with two cloned receptors that were originally differentiated on the basis of their affinity to the antihistaminic drug levocabastine (Schotte et al. 1986). The high sensitive, levocabastine-insensitive rat neurotensin receptor (*NTR1*) was cloned first (Tanaka et al. 1990) and shown to mediate a number of peripheral and central neurotensin responses, including the neuroleptic-like effects of the peptide (Labbé-Jullié et al. 1994). The human *NTR1* has been cloned from the colonic adenocarcinoma cell line HT29 (Vita et al. 1993) and shown to consist of a 416 amino acid protein that shares 84% homology with rat *NTR1*. A second human *NTR1* receptor differing only in one amino acid has been cloned from substantia nigra by Watson et al. (1993).

The lower-affinity, levocabastine-sensitive neurotensin receptor (*NTR2*) was cloned by Chalon et al. (1996), Mazella et al. (1996) and characterized by Yamada et al. (1998). Studies by Dubuc et al. (1999) indicate that *NTR2* mediates neurotensin-induced analgesia.

A third neurotensin receptor (*NTR3*) was cloned from a human brain cDNA library (Mazella et al. 1998; Vincent et al. 1999; Mazella 2001; Mazella and Vincent 2006). It is identical with sortilin, a receptor-like protein, cloned from human brain (Petersen et al. 1997, 1999). The NT3/gp95/sortilin protein is a transmembrane neuropeptide receptor which does not belong to the superfamily of G-protein-coupled receptors.

Gully et al. (1997) described a binding assay for the neurotensin1 receptor.

PROCEDURE

Cell Culture

CHO cells transfected with cDNA of the human neurotensin receptor cloned from HT 29 cells (h-NTR1-CHO cells) are cultured at 37°C in modified Eagles medium without nucleosides, containing 10% fetal calf serum, 4 mM glutamine and 300 µg/ml geneticin (G418), in a humidified incubator under 5% CO₂ in O₂. The colonic adenocarcinoma HT 29 cell line (American Type Culture Collection, Rockville, MD) is cultured under similar conditions in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal calf serum, 4 mM glutamine, 200 IU/ml penicillin and 200 mg/ml streptomycin. One week after seeding, confluent monolayer cultures are washed three times with 3 ml PBS and harvested by enzymatic dissociation with trypsin. After dilution with PBS, cells are resuspended in the same culture medium at a density of 5 × 10⁴ cells/ml and are plated into 35-mm-diameter, fibronectin-coated Petri culture dishes.

Membrane Homogenate Preparation and Binding Assay

Whole brains of male Sprague-Dawley rats albino guinea pigs or cell pellets are homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) for 30 s, using a Polytron homogenizer (setting 5). After 20 min centrifugation at 30,000 g, the pellet is washed, centrifuged again under the same conditions, resuspended in a storage buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% BSA, 40 mg/liter bacitracin, 1 mM 1,10-orthophenanthroline and 5 mM dithiothreitol and stored as aliquots in liquid nitrogen until used.

Aliquots of membranes (10, 50, 300 and 500 µg of protein for h-NTR1-CHO cells, HT 29 cells, rat

and guinea pig brain, respectively) are incubated for 20 min at 20°C in the incubation buffer (0.5 ml final volume) containing appropriate concentrations of [¹²⁵I-Tyr³]neurotensin (25–100 pM) and unlabeled drugs. After incubation, the assay medium is diluted with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.1% BSA and 1 mM EDTA, and the mixture is rapidly filtered under reduced vacuum through Whatman GF/B glass fibre filters that have been pretreated with 0.1% polyethyleneimine. The filters are washed under the same conditions three times and radioactivity is measured. Nonspecific binding is determined in the presence of 1 µM unlabeled neurotensin. All experiments are performed in triplicate, and data are expressed as the mean ± SEM of at least three separate determinations.

EVALUATION

The IC₅₀ is the value of ligand that inhibits 50% of the specific binding and is determined using an iterative nonlinear regression program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

Cusack et al. (1995) studied species selectivity of neurotensin analogs at the rat and two human *NTR1* receptors.

Lugrin et al. (1991) produced a series of pseudopeptide analogs of neurotensin by systematically replacing peptide bonds in neurotensin with CH₂NH bonds. The compounds were screened *in vitro* for agonist or antagonist activity and for metabolic stability.

Le et al. (1997) cloned the human neurotensin receptor gene and determined the structure.

Labbé-Jullié et al. (1998) attempted to identify residues in the rat *NTR1* that are involved in binding of a nonpeptide neurotensin antagonist.

Souazé et al. (1997), Najimi et al. (1998) studied the effects of a neurotensin agonist and showed in human colonic adenocarcinoma HT 29 cells after short incubation an increase, after prolonged exposure a decrease of mRNA levels and in the human neuroblastoma cell line CHP 212 a high affinity neurotensin receptor gene activation.

Ovigne et al. (1998) described a monoclonal antibody specific for the human *NTR1*.

Nouel et al. (1999) found that both NT2 and NT3 neurotensin receptor subtypes were expressed by cortical glial cells in culture.

Cusack et al. (2000) developed a neurotensin analog, NT34, that can distinguish between rat and human

neurotensin receptors, and exhibits more than a 100-fold difference in binding affinities.

Neuromedin N, a peptide belonging to the gastrin-releasing peptide/bombesin family (see J.3.1.7) shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1990).

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E.5.1.17

Genetically Altered Monoamine Transporters

Monoamine transporters, such as the dopamine transporter, 5-hydroxytryptamine transporter and noradrenaline transporter, in the plasma membrane pro-

vide effective control over the intensity of monoamine-mediated signaling by recapturing neurotransmitters released by presynaptic neurons (Gainetdinov et al. 2002). These transporters act also as molecular gateways for neurotoxins (Uhl and Kiyama 1993; Miller et al. 1999; Vincent et al. 1999).

Takahashi et al. (1997) found that heterozygote animals of VMAT2 knockout mice display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity.

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E.5.1.17.1

Dopamine Transporter Knockout Mice

Many drugs exert their psychotropic action via dopamine transporters (Amara and Kuhar 1993; Giros and Caron 1993).

Dopamine transporter knockout mice, which are generated by disruption of the gene encoding the dopamine transporter by homologous recombination (Giros et al. 1996; Sora et al. 1998), have a distinct biochemical and behavioral phenotype. At the neurochemical level, the homeostasis of dopamine-containing neurons is altered markedly, including disrupted clearance of dopamine, an elevated extracellular concentration of dopamine and dramatically decreased intraneuronal storage of dopamine (Jones et al. 1998; Gainetdinov et al. 1998; Benoit-Marand et al. 2000).

In response to the elevated dopamine-mediated tone, both presynaptic and postsynaptic dopamine receptors are downregulated (Giros et al. 1996), but although autoreceptor functions are lost (Jones et al. 1999), some postsynaptic responses appear to be enhanced (Gainetdinov et al. 1999a; Fauchey et al. 2000).

Dopamine transporter knockout mice are hyperactive (Gainetdinov et al. 1999b; Spieleswoy et al. 2000) and have a much reduced body size (Bossé et al. 1997). These animals have cognitive deficits (Gainetdinov et al. 1999a, 1999b), disrupted sensorimotor gating (Ralph et al. 2001) and sleep dysregulation (Wisor et al. 2001). Dopamine transporter knockout mice appear to provide a model of some aspects of manic behavior (Ralph-Williams et al. 2003).

Abnormalities in skeletal structure (Blizotes et al. 2000) and altered regulation of gastrointestinal tract motility (Walker et al. 2000) are also observed.

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E.5.1.17.2

Serotonin Transporter Knockout Mice

The serotonin transporter has a key role in regulating the intensity of 5-HT-mediated transmission and is the primary target for several antidepressants and psychostimulants (Amara and Kuhar 1993; Bengel et al. 1996).

Disruption of 5-HT uptake in serotonin transporter knockout mice increases the extracellular concentration of 5-HT sixfold and reduces intracellular concentration by 60%–80% (Fabre et al. 2000).

Holmes et al. (2003) found that mice lacking the serotonin transporter exhibit 5HT_{1A} receptor-mediated abnormalities in tests for anxiety-like behavior.

Lira et al. (2003) reported altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin-transporter-deficient mice.

Marked desensitization of both presynaptic and postsynaptic 5-HT_{1A} receptors is observed in electrophysiological studies (Gobbi et al. 2001).

There is a significant decrease in 5-HT_{1A} receptor binding sites, mRNA and protein in some, but not all, 5-HT-containing brain areas. Altered hypothalamic and neuroendocrine responses to 8-hydroxy-2-(di-n-propylamino)-tetraline (8-OH-DPAT) are also reported (Li et al. 1999).

Thermal hyperalgesia in mice after chronic constrictive sciatic nerve injury was absent in serotonin-transporter-deficient mice (Vogel et al. 2003).

Decreases in 5-HT_{1A} and 5-HT_{1B} receptor coupling are observed, accompanied by disruption of the neurochemical responses to the 5-HT_{1A} receptor agonist ipsapirone and the 5-HT_{1A}/5-HT_{1D} receptor agonist GR127935 (Fabre et al. 2000).

The hyperlocomotor effect of MDMA, but not that of high doses of d-amphetamine, is disrupted in serotonin receptor knockout mice (Bengel et al. 1996).

In double knockout mice that lack the dopamine transporter and have no or one copy of the gene that encodes the serotonin transporter, no place preference for cocaine was observed (Sora et al. 2001).

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E.5.1.17.3

Noradrenaline Transporter Knockout Mice

The noradrenaline transporter has a role similar to that of the dopamine transporter and the serotonin transporter with respect to noradrenaline-mediated transmission (Blakely et al. 1994).

Noradrenaline-transporter knockout mice have been generated using homologous recombination (Xu et al. 2000).

Wang et al. (1999) reviewed genetic approaches to studying norepinephrine function using knockout of the mouse norepinephrine transporter gene.

The prolonged synaptic lifetime of noradrenaline in noradrenaline transporter knockout mice results in elevation of the extracellular concentration of noradrenaline and depletion of the intraneuronal stores. In addition, in noradrenaline transporter knockout mice, the α_1 -adrenoceptor decreased in the hippocampus (Xu et al. 2000), although α_{2A} -adrenoceptor density did not change in the spinal cord (Bohn et al. 2000).

Noradrenaline-transporter knockout mice have a lower body weight and reduced locomotor responses to novelty. In the tail-suspension test used for screening antidepressant drugs, noradrenaline transporter knockout mice behaved like antidepressant-treated, wild-type animals and no additional effects of the antidepressants desimipramine, paroxetine and bupropion were observed in mutant mice in this test (Xu et al. 2000).

In the tail-flick assay, morphine induced greater analgesia in noradrenaline transporter knockout mice compared with wild-type mice (Bohn et al. 2000).

In synaptosomes from the frontal cortex of noradrenaline transporter knockout mice, cocaine and nisoxetine had no inhibitory effect on the uptake of dopamine, whereas in the nucleus accumbens the effectiveness of cocaine was somewhat reduced. Uptake of dopamine in brain regions that have low levels of dopamine transporter may depend primarily on the noradrenaline transporter (Morón et al. 2002).

Locomotor responses to cocaine and amphetamine are elevated in noradrenaline transporter knockout mice and chronic administration of cocaine did not induce further sensitization. The enhanced responses to psychostimulants in noradrenaline transporter knockout mice correlate with the suppression of presynaptic dopamine function and supersensitivity to postsynaptic D2 and D3 receptors (Xu et al. 2000).

Haller et al. (2002) studied behavioral responses to social stress in noradrenaline transporter knockout mice.

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E.5.2

In Vivo Tests

E.5.2.1

Golden Hamster Test

PURPOSE AND RATIONALE

“Innate behavior” of many species including man has been described by Lorenz (1943, 1966). The “Golden-hamster-test” (Ther, Vogel and Werner 1959) uses the innate behavior of this species (*Mesocricetus auratus*) for differentiation between neuroleptic and sedative – hypnotic activity. The aggressive behavior of male golden hamsters is suppressed by neuroleptics in doses which do not impair motor function.

PROCEDURE

Ten to 20 male golden hamsters with an average weight of 60 g are crowded together in Makrolon^(R) cages for at least 2 weeks. During this time the animals develop a characteristic fighting behavior. For the test single animals are placed into glass jars of 2 liters. In this situation the hamsters assume a squatting and resting position during the day. If the animals are touched with a stick or a forceps they wake up from their daytime sleep and arouse immediately from the resting position. If one tries to hold the hamster with a blunted forceps, a characteristic behavior is elicited: The hamster throws himself onto his back, tries to bite and to push the forceps away with his legs, and utters angry shrieks. Touching the animals is repeated up to 6 times followed by punching with the forceps. Only animals responding to the stimulus with all three defense reactions (turning, vocalizing, biting) are included into the test.

The test compounds are applied either subcutaneously, intraperitoneally, or orally. Six animals are used for each dose.

EVALUATION

The stimuli are applied every 20 min for 3 h. The number of stimuli until response is recorded. Furthermore, the suppression of the defense reactions (turning, biting and vocalizing) is evaluated. An animal is regarded to be completely "tamed", if all defense reactions are suppressed even after punching with the forceps at least once during the test period.

After each stimulation the "tamed" animal is placed on an inclined board with 20 degree inclination. Normal hamsters and hamsters tamed by neuroleptics are able to support themselves or to climb on the board. Impaired motor function causes sliding down. This experiment is repeated three times after each testing of the defense reactions. An animal's coordination is considered to be disturbed if it falls three times during two tests of the experiments.

For each dose the number of tamed hamsters and the number of animals with impaired motor function is recorded. Using different doses, ED_{50} values can be calculated for the taming effect and for impairment of motor function.

The ED_{50} values of taming were 1.5 mg/kg for chlorpromazine s.c. and 0.2 mg/kg for reserpine s.c. Much higher doses (10 times of chlorpromazine and 5 times of reserpine) did not elicit motor disturbances. On the contrary, while ED_{50} values of 10 mg/kg phenobarbital s.c. and 180 mg/kg meprobamate p.o. for the taming effect were found, these doses already caused severe motor disturbances. The taming dose of diazepam was 10 mg/kg p.o. which already showed some muscle relaxing activity. The term "neuroleptic width" indicates the ratio between the ED_{50} for taming and the ED_{50} for motor disturbances. Only for neuroleptic drugs are ratios found between 1:5 and 1:30.

CRITICAL ASSESSMENT OF THE METHOD

The method has the advantage that neuroleptics can easily be differentiated from sedative and hypnotic drugs. Anxiolytics with pronounced muscle relaxing activity also show no significant differences between taming and impaired motor function. Moreover, the method has the advantage that no training of the animals and no expensive apparatus is needed.

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E.5.2.2

Influence on Behavior of the Cotton Rat

PURPOSE AND RATIONALE

The "cotton-rat test" is another attempt to use the innate behavior as described for several animal species by Lorenz (1943, 1966) for the differentiation of psychotropic drugs (Vogel and Ther 1960). The cotton rat (*Sigmodon hispidus*) is a very shy animal which conceals himself at any time. This innate flight reflex is suppressed by centrally active drugs. Simultaneous evaluation of motor function allows the differentiation between neuroleptic and sedative drugs.

PROCEDURE

Cotton rats are bred in cages equipped with a clay cylinder of 20 cm length and 10 cm diameter. This cylinder is used by the animals for hiding, sleeping and breeding. Moreover, the animals which bite easily can be transported from one cage to another just by closing the cylinder on both ends. For the test young animals with a body weight of 40 g are used. Young animals are as shy as old ones but less vicious. Nevertheless, leather gloves have to be used for handling of cotton rats. Normal cages (25 × 30 × 20 cm) with a wire lid are used. A tunnel of sheet metal (half of a cylinder) 20 cm long and 7 cm high is placed into the cage. The cotton rats hide immediately in this tunnel. If the tunnel is lifted and placed on another site of the cage, the cotton rats immediately hide again.

Three rats are placed in one cage and tested for their behavior. Selective shaving of the fur enables the observer to recognize each animal. If the rats behave as described, they are then treated with the test compound subcutaneously or orally. At least 6 animals divided in two cages are used for each dose of test compound or standard. Fifteen min after application of the drug the test period of three h is started. The tunnel is lifted and placed to another site. If the animals do not show the immediate flight reflex an airstream of short duration is blown through the wire lid. If the animal still does not respond with the flight reflex it is considered to be positively influenced. Afterwards, the animal is placed on an inclined board with 35 degree of inclination and tested for disturbance of motor coordination. A normal animal is able to climb upwards. If coordination is disturbed the rat slides down.

EVALUATION

The test procedure is repeated every 15 min over a period of 3 h. The animals which show at least one suppression of the flight reflex during the test period are counted as well as those who slide down on the inclined board. Using different doses ED_{50} values are calculated for both parameters. The ratio between these two ED_{50} values is regarded as "neuroleptic width" which is 1:20 for chlorpromazine and 1:30 for reserpine whereas ratios of 1:2 for phenobarbital and 1:1.5 for meprobamate indicate the absence of neuroleptic activity.

CRITICAL ASSESSMENT OF THE METHOD

The method allows the differentiation of drugs with neuroleptic activity against other centrally active drugs. No training of the animals and no expensive equipment are necessary.

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E.5.2.3**Artificial Hibernation in Rats****PURPOSE AND RATIONALE**

Giaja (1938, 1940, 1953, 1954) studied the effects of reduced oxygen tension and cold environment on rats. The animals were placed in hermetically closed glass vessels which were submerged in ice-water. Due to the respiratory activity, the oxygen tension diminishes and the carbon dioxide content increases. Under the influence of cooling and of hypoxic hypercapnia, the rectal temperature falls to 15°C and the animal is completely anesthetized and immobilized. The rat can survive in this poikilothermic state for more than twenty hours. Complete recovery occurs after warming up. This kind of artificial hibernation was augmented by chlorpromazine (Courvoisier et al. 1953; Giaja and Markovic-Giaja 1954). Vogel (1959), Ther et al. (1959, 1963) used these observations for evaluation of neuroleptics and opioid analgesics.

PROCEDURE

Male Wistar rats weighing 100–150 g are deprived of food with free access to tap water overnight. The test compounds are injected subcutaneously 15 min prior

to the start of the experiment. First, the rats are placed in ice-cold water to which surfactant is added in order to remove the air from the fur for 2 min. Then, the animals are placed into hermetically closed glass vessels of 750 ml volume which are placed into a refrigerator at 2°C temperature. During the following hour, the vessels are opened every 10 min for exactly 10 s allowing some exchange of air and reducing the carbon dioxide accumulation. At each time, animals are removed from the glass vessel and observed for signs of artificial hibernation which are not shown by control animals under these conditions. Treated animals, lying on the side, are placed on the back and further examined. An animal is considered positive, when it remains on the back, even if the extremities are stretched out. In this state, cardiac and respiration frequency are reduced and the rectal temperature has fallen to 12–15°C. The rigor of the musculature allows only slow movements of the extremities. The animals recover completely within a few hours if they are brought to their home cages at room temperature. Artificial hibernation is induced dose-dependent by neuroleptics of the phenothiazine type and by some opioid analgesics like meperidine and methadone. In contrast, morphine shows only slight activity.

EVALUATION

Various doses are applied to groups of 10 animals. Percentage of positive animals is calculated for each group and ED_{50} values with confidence limits are estimated according to Litchfield and Wilcoxon.

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E.5.2.4

Catalepsy in Rodents

PURPOSE AND RATIONALE

Catalepsy in rats is defined as a failure to correct an externally imposed, unusual posture over a prolonged period of time. Neuroleptics which have an inhibitory action on the nigrostriatal dopamine system induce catalepsy (Costall and Naylor 1974; Chermat and Simon 1975; Sanberg 1980) while neuroleptics with little or no nigrostriatal blockade produce relatively little or no cataleptic behavior (Honma and Fukushima 1976). Furthermore, cataleptic symptoms in rodents have been compared to the Parkinson-like extrapyramidal side effects seen clinically with administration of antipsychotic drugs (Duvoisin 1976).

PROCEDURE

Groups of 6 male Sprague-Dawley or Wistar rats with a body weight between 120 and 250 g are used. They are dosed intraperitoneally with the test drug or the standard. Then, they are placed individually into translucent plastic boxes with a wooden dowel mounted horizontally 10 cm from the floor and 4 cm from one end of the box. The floor of the box is covered with approximately 2 cm of bedding material. White noise is presented during the test. The animals are allowed to adapt to the box for 2 min. Then, each animal is grasped gently around the shoulders and under the forepaws and placed carefully on the dowel. The amount of time spent with at least one forepaw on the bar is determined. When the animal removes its paws, the time is recorded and the rat is repositioned on the bar. Three trials are conducted for each animal at 30, 60, 120 and 360 min.

EVALUATION

An animal is considered to be cataleptic if it remains on the bar for 60 s. Percentage of cataleptic animals is calculated. For dose-response curves, the test is repeated with various doses and more animals. ED_{50} values can be calculated. A dose of 1 mg/kg i.p. of haloperidol was found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The phenomenon of catalepsy can be used for measuring the efficacy and the potential side effects of neuroleptics.

MODIFICATIONS OF THE METHOD

Catalepsy induced by neuroleptic drugs can also be measured by the **PAW test**, which measures increase in forelimb and hindlimb retraction time in rats (Ellenbroek et al. 1987, 2001; Ellenbroek and Cools 1988, 2000; Prinszen et al. 1994, 1995).

The test is performed 30 min after intraperitoneal injection of test drug. Male Wistar rats weighing 220–300 g are placed on a Perspex platform (30 × 30 cm with a height of 20 cm) containing two holes for the forelimbs (40 mm) and two for the hindlimbs (50 mm), and a slit for the tail. The distance between the right and left forelimb holes is 15 mm, and the distance between forelimb and hindlimb holes is 55 mm. The rat is held behind the forelimbs, and the hindlimbs are gently placed in the holes. The forelimb retraction time and the hindlimb retraction time are defined as the time the animal needs to withdraw one forelimb and one hindlimb, respectively. The average forelimb retraction time and hindlimb retraction time (the mean of three measurements) is calculated for each rat.

Extrapyramidal syndromes after treatment with typical and atypical neuroleptics were measured in nonhuman primates (Cebus monkeys) by Casey (1989, 1991, 1993), Gerlach and Casey (1990).

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E.5.2.5

Pole Climb Avoidance in Rats

PURPOSE AND RATIONALE

The pole-climb avoidance paradigm is an avoidance-escape procedure used to separate neuroleptics from sedatives and anxiolytics. Whereas sedative compounds suppress both avoidance and escape responding at approximately the same doses, neuroleptic drugs reduce avoidance responding at lower doses than those affecting escape responding (Cook and Catania 1964).

PROCEDURE

Male rats of the Long-Evans strain with a starting body weight of 250 g are used. The training and testing of the rats is conducted in a 25 × 25 × 40 cm chamber that is enclosed in a dimly lit, sound-attenuating box. Scrambled shock is delivered to the grid floor of the chamber. A 2.8-kHz speaker and a 28-V light are situated on top of the chamber. A smooth stainless-steel pole, 2.5 cm in diameter, is suspended by a counterbalance weight through a hole in the upper center of the chamber. A microswitch is activated when the pole is pulled down 3 mm by a weight greater than 200 g. A response is recorded when a rat jumps on the pole and activates the microswitch. The rat can not hold the pole down while standing on the grid floor because

of the counterbalance tension and can not remain on the pole any length of time because of its smooth surface. The activation of the light and the speaker together are used as the conditioning stimulus. The conditioning stimulus is presented alone for 4 s and then is coincident with the unconditioned stimulus, a scrambled shock delivered to the grid floor, for 26 s. The shock current is maintained at 1.5 mA. A pole climb response during the conditioned stimulus period terminates the conditioned stimulus and the subsequent conditioned and unconditioned stimuli. This is considered an avoidance response. A response during the time when both the conditioned and unconditioned stimuli are present terminates both stimuli and is considered an escape response. Test sessions consist of 25 trials or 60 min, whichever comes first. There is a minimum intertrial interval of 90 s. Any time remaining in the 30 s allotted to make the pole climb is added to the 90 s intertrial interval. Responses during this time have no scheduled consequences; however, rats having greater than 10 intertrial interval responses should not be used in the experiment. Before testing experimental compounds, rats are required to make at least 80% avoidance responses without any escape failures.

EVALUATION

Data are expressed in terms of the number of avoidance and escape failures relative to the respective vehicle control data. *ED*₅₀ values can be calculated using different doses.

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E.5.2.6

Foot-Shock Induced Aggression

PURPOSE AND RATIONALE

The test as described by Tedeschi et al. (1959) using mice which fight after foot-shock induced stimulation

is useful to detect neuroleptics but also shows positive effects with anxiolytics and other centrally effective drugs. The method has been used by several authors to test drugs with neuroleptic activity. The test is described in Sect. E.2.3.1.

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E.5.2.7

Brain Self Stimulation

PURPOSE AND RATIONALE

In several species, electrical stimulation of selected brain loci produces effects which are positively reinforcing and pleasurable (Olds and Milner 1954; Olds 1961, 1972). Most of the data available have been obtained from experiments using rats with electrodes chronically implanted in the median forebrain bundle at the level of hypothalamus. Minute electrical pulses sustain a variety of operant behaviors such as lever pressing. Neuroleptics have been shown to be potent blockers of self stimulation (Broekkamp and Van Rossum 1975; Koob et al. 1978; Gallistel and Freyd 1987). Conversely, compounds that facilitate catecholaminergic transmission such as d-amphetamine and methylphenidate, will increase responding for such stimulation.

PROCEDURE

Male Wistar rats (350–400 g) are anesthetized with 50 mg/kg pentobarbital i.p. and their heads placed on a level plane in a Kopf stereotactic instrument. A mid-line incision is made in the scalp and the skin held out of the way by muscle retractors. A small hole is drilled in the skull with a dental burr at the point indicated by the stereotactic instrument for the structure it is desired to stimulate. Using bregma as a reference point, the electrode (Plastic Products MS303/1) is aimed at the median forebrain bundle according to the atlas of Paxinos and Watson (1986), using the coordinates of AP = -0.8 mm, Lat = +2.8 mm, and DV = -7.2 mm below dura. The assembly is then permanently affixed to the skull using stainless steel screws and bone cement.

After a minimum of 10 days for recovery, the animals are trained to bar press for electrical stimulation on a continuous reinforcement schedule in a standard operant box outfitted with a single lever. The reward stimulus is a train of biphasic square-wave pulses generated by a Haer stimulator (Pulsar 4i). The parameters

are set at a pulse duration of 0.5 ms with 2.5 ms between each pulse pair. The train of pulses may vary between 16 and 30/s, and the intensity of the pulses that are delivered range from 0.1 to 0.5 mA using the lowest setting that will sustain maximal responding. After consistent baseline responding is obtained for 5 consecutive 30 min sessions, the animals are ready for testing with standard agents. Compounds are administered 60 min prior to testing. All data are collected on both cumulative recorders and counters.

EVALUATION

The number of drug responses are compared to the number of responses made during each animal's 30 min control session on the preceding day, which is considered to be equal to 100%. Testing various doses, ED_{50} values with 95% confidence limits can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

Since there is sufficient evidence that self-stimulation behavior is maintained by catecholamines the method gives indirectly insight into the catecholaminergic facilitating or blocking properties of a compound. Active neuroleptic drugs inhibit the self-stimulation behavior in very small doses. The relative potency observed in this test of clinically efficacious drugs parallels their potency in the treatment of schizophrenia.

MODIFICATIONS OF THE METHOD

Reinforcing brain stimulation by electrodes placed in the medial forebrain bundle of rats is decreased after lesion of the internal capsule in the region of the diencephalic-telencephalic border. This decrement in rewarding processing can be reversed by antidepressant drugs (Cornfeldt et al. 1982).

Depoortere et al. (1996) used electrical self stimulation of the ventral tegmental area to study the behavioral effects of a putative dopamine D_3 agonist in the rat.

Anderson et al. (1995) examined the interaction of aversive and rewarding stimuli in self stimulating rats in terms of duration and direction. The rats were implanted with two moveable electrodes, one in a region supporting self-stimulation (the ventral tegmental area) and another in a region supporting escape (the nucleus reticularis gigantocellularis).

Kokkinidis et al. (1986) used amphetamine withdrawal for a behavioral evaluation. Mice implanted with stimulating electrodes in the lateral hypothalamus demonstrated stable and reliable rates of self-stimulation responding. After exposure to a chronic sched-

ule of amphetamine treatment response rates were severely depressed.

Post-amphetamine depression of self-stimulation from the substantia nigra can be reversed by cyclic antidepressants (Kokkinidis et al. 1980).

Moreau et al. (1992) reported that antidepressant treatment prevents chronic unpredictable mild stress-induced anhedonia as assessed by ventral tegmentum self-stimulation in rats.

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E.5.2.8

Prepulse Inhibition of Startle Response

PURPOSE AND RATIONALE

Prepulse inhibition is a model of sensorimotor gating which can be assessed in both animals and humans using the startle reflex response. When a fixed startle eliciting stimulus (i.e., the pulse) is preceded by 30–500 ms by a weak, non-startle-eliciting stimulus (i.e., the prepulse), the magnitude of the startle response is significantly reduced to the pulse alone. Schizophrenic patients have decreased prepulse inhibition relative to normal control subjects, and this is thought to reflect an impairment in their ability to filter irrelevant sensory stimuli (Braff and Geyer 1990; Geyer 1998). Similar reductions in prepulse inhibition are produced in rats by administration of psychotomimetic drugs such as the dopamine agonists amphetamine and apomorphine or the non-competitive NMDA antagonists phencyclidine and dizocilpine (MK801) (Mansbach and Geyer 1989; Swerlow et al. 1998; Geyer et al. 2001; Rowley et al. 2001; Weiss and Feldon 2001; Pouzet et al. 2002). Most antipsychotics tested are able to antagonize prepulse inhibition disruption produced by dopamine antagonists, whereas prepulse inhibition disruption by NMDA antagonists may be selectively sensitive to antipsychotics with atypical features (Bakshi and Geyer 1995; Bubenikova

et al. 2005; Fox et al. 2005). Haloperidol failed to block the effects of phencyclidine and dizocilpine prepulse inhibition of startle (Keith et al. 1991).

Feifel et al. (Feifel and Reza 1999; Feifel et al. 1999a, 1999b) tested the effects of a neurotensin agonist on prepulse inhibition of startle in rats.

PROCEDURE

Male Sprague Dawley rats were treated with various doses of test compound or saline s.c. Immediately afterwards, rats receive a second s.c. injection consisting of 2 mg/kg d-amphetamine, or 0.5 mg/kg apomorphine, or 0.1 mg/kg dizocilpine or saline. Then, 10 min later, animals were placed in special startle chambers (SR-LAB, San Diego Instruments, San Diego, Calif., USA). Startle chambers consist of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5 × 25.5 cm Plexiglas frame within a ventilated enclosure housed in a sound-attenuated room exposed to 70-dB background noise. After a 5-min acclimation period, acoustic stimuli were presented via a speaker mounted 24 cm above the animal. Acoustic stimuli consisted of a 120-dB pulse by itself (pulse alone) or a 120-dB pulse preceded by 100 ms by prepulses 3, 5, and 10 dB above background noise. There was an average of 15 s between stimuli. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced the motion within the cylinder. Startle amplitude was defined as the degree of motion detected by this accelerometer. Each rat was tested on four separate occasions separated by 7 non-test days. On each test day, the dose of test compound was kept constant, but the specific psychotomimetic agent was alternated across test days in a counterbalanced fashion.

EVALUATION

Prepulse inhibition was calculated as the percentage of the pulse-alone startle amplitude using the following formula: $[1 - (\text{startle amplitude after prepulse-pulse pair} / \text{startle amplitude after pulse only})] \times 100$. Analysis of data was then carried out using a three-factor repeated-measures analysis of variance (ANOVA). Significant factor results from the ANOVA were followed up with separate one-way ANOVAs for each psychotomimetic agent and then, when indicated, with individual group mean comparisons using post hoc *t* tests for multiple comparisons using the Bonferroni method.

MODIFICATIONS OF THE METHOD

Sipes and Geyer (1995) studied the disruption of prepulse inhibition of the startle response in the rat by

DOI [(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride], which is mediated by 5-HT_{2A} receptors. The authors suggested that studies of the serotonergic substrates of prepulse inhibition may provide a model of the possible serotonergic role in the sensorimotor gating abnormalities in patients with schizophrenia and with obsessive compulsive disorder.

Ellenbroeck et al. (1998) described the effects of an early stressful life event on sensorimotor gating in adult rats.

Andersen and Pouzet (2001) compared the effects of acute versus chronic treatment with typical or atypical antipsychotics on d-amphetamine-induced sensorimotor gating deficits in rats.

Heidbreder et al. (2000) used the prepulse inhibition of acoustic startle for behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome.

Krebs-Thomson et al. (2001) reported that post-weanling handling attenuates isolation-rearing disruption of prepulse inhibition in rats.

Weiss et al. (2001) studied the dissociation between the effects of pre-weaning and/or post-weaning social isolation on prepulse inhibition and latent inhibition in adult Sprague Dawley rats.

Dirks et al. (2003) reported reversal of startle gating deficits in transgenic **mouse** overexpressing corticotropin-releasing factor by antipsychotic drugs.

Andreasen et al. (2006) studied the effect of nicotinic agents on prepulse inhibition (PPI) in mice using a startle response/PPI system from TSE Systems, Bad Homburg, Germany.

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E.5.2.9

N40 Sensory Gating

PURPOSE AND RATIONALE

The N40 auditory-evoked potential has been used to develop an animal model for the study of sensory gating mechanisms (Boutros et al. 1997a; Boutros and Kwan 1998). The method has been applied to evaluation of psychotropic compounds (Adler et al. 1986; Boutros et al. 1994, 1997b). Bickford-Wimer et al. (1990) localized one possible source of the N40 waveform to the CA3 region of the hippocampus.

Fox et al. (2005) used the N40 sensory gating model in mice for evaluation of a potential antipsychotic drug.

PROCEDURE

Male DBA/2 mice were stereotaxically implanted with tripolar stainless steel wire head stages for EEG recordings in the CA3 region of the hippocampus. The mice were first anesthetized with a solution of 2.8% ketamine, 0.28% xylazine, and 0.05% acepromazine. Three access holes for the electrodes were made at AP –1.8 mm from the bregma, and in a plane perpendicular to the suture, ML 0.6 (cortical electrode), 1.6 (reference electrode), and 2.6 mm electrode directed at the hippocampus). The depth of the hippocampal electrode tip was DV 1.65–1.70 mm below the surface of the cortex. The depths of the cortical and reference electrodes were DV 0.5 mm from the surface of the skull, resulting in contact, but not penetration of the cortical tissue. The tripolar electrode was lowered into position with a stereotaxic electrode holder and affixed using cyanoacrylic gel and dental acrylic and two anchor screws. Mice were allowed to recover for 3 days before commencement of the experiments. Awake mice were recorded in acoustically isolated chambers. Flexible tethers and electrical swivels were used to convey EEG signals to differential AC EEG amplifiers and allowed the mice free movement within the chambers. The EEG was amplified 1000× with

a 50- to 60-Hz notch filter engaged, and high- and low-pass filters were set at 1 and 100 Hz, respectively. Hippocampal auditory-evoked potentials were generated by presentation of 60 sets of 3 kHz paired tone bursts from a speaker within the recording chamber at a distance of 15–20 cm to the mouse. The first tone of the pair is referred to as the conditioning stimulus, and the second is referred to as the test stimulus. The duration of both the condition and test stimuli was 5 ms, with 0.5 s between the stimuli and 20 s between pairs. Data acquisition software recorded EEG signals 100 ms before and for 899 ms after the initial conditioning stimulus. The software averaged the 60 paired responses into one composite-evoked response. Various doses of test drug were administered i.p. 20–30 min before mice were placed into the recording chambers and initiation of auditory-evoked potential recording. Recording of paired auditory potentials continued for two 20-min sessions, each comprised of 60 paired stimuli. Each mouse was administered every treatment dose and a control vehicle treatment in a balanced order on separate days with at least 48 h between treatments. This within-subject design allowed each mouse to serve as its own control. The hippocampal response to auditory stimuli was identified as the highest positive peak deflection in the ongoing EEG at a latency of 10–20 ms after the stimulus (P20), followed by the lowest negative peak deflection in the ongoing EEG at 20–45 ms after the stimulus (N40). The difference in amplitude between P20 and N40 was defined as the N40 amplitude in microvolts.

EVALUATION

N40 amplitudes were determined for both the averaged conditioning and test-evoked potentials, and a ratio was derived between the two responses by dividing the test amplitude by the conditioning amplitude (T/C ratio).

MODIFICATIONS OF THE METHOD

Flack et al. (1996) studied sensory gating in a computer model of the CA3 neural network of the hippocampus.

Stevens et al. (1998) investigated changes in auditory information processing after kainic acid lesions in adult rats used as a model of schizophrenia.

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E.5.2.10

Latent Inhibition

PURPOSE AND RATIONALE

Latent inhibition has been recommended as an animal model of schizophrenia (Feldon and Weiner 1992; Swerdlow et al. 1996; Vaitl and Lipp 1997; Moser et al. 2000; Bender et al. 2001). Latent inhibition refers to the retarded acquisition of a conditioned response that occurs if the subject being tested is first pre-exposed to the to-be-conditioned stimulus without the paired unconditioned stimulus. Because the “irrelevance” of the to-be-conditioned stimulus is established during non-contingent pre-exposure, the slowed acquisition of the conditioned stimulus–unconditioned stimulus association is thought to reflect the process of overcoming this learned irrelevance. Latent inhibition has been reported to be diminished in acutely hospitalized schizophrenia patients. Several authors used the latent inhibition model in rats to test psychotropic compounds (Solomon et al. 1981; Feldon and Weiner 1991; Moran and Moser 1992; De la Casa et al. 1993; Lacroix et al. 2000; Alves et al. 2002). Trimble et al. (2002) tested the effects of selective D₁ antagonists on latent inhibition in the rat.

PROCEDURE**Animals**

Male Sprague Dawley rats weighing 300–400g were housed two to a cage under a 12-h reversed cycle lighting with food and water ad libitum. All experimental manipulations were carried out in the dark phase of the dark/light cycle.

Apparatus

Modified metal Skinner boxes (24.5 × 24.5 × 21 cm measured from a raised grid floor) were located in darkened, sound-insulated, ventilated outer boxes. A removable water bottle was located on one side of each Skinner box through a hole of 1.0 cm diameter, positioned 2 cm above the grid floor. When water was not required, the water bottle was removed. Licks at the spout of each water bottle were recorded using a lickometer (model 453, Campden Instruments, London, UK). The pre-exposed stimulus was a flashing light (10 s duration with three light flashes per second) situated in the middle of the roof of each Skinner box. The grid floor consisted of steel bars (0.5 cm in diameter) spaced 1 cm apart. Shock generators with scramblers were calibrated to produce 0.5-mA shocks via the grid floor.

Procedure

Rats were randomly assigned to experimental groups and were allocated to a particular Skinner box. They had experience of only that box for the duration of the experiment. After adaptation to the housing conditions for 1 week, rats were placed immediately on a 23-h water deprivation schedule that continued until the end of the experiments. Food remained freely available.

Baseline days (days 15–19)

After 7 days on the water deprivation period, 5 days of pre-training commenced. Each rat was placed in a Skinner box for 15 min. The water bottle was present and each rat could drink freely. After the baseline session was over, each rat was returned to its home cage and allowed access to water for 45 min.

Pre-exposure (day 20)

With the water bottle removed, each rat was placed in a Skinner box. Rats received ten stimulus (flashing house-light) presentations of 10 s duration (three light flashes per second) with a fixed stimulus interval of 50 s. Afterwards the rats were returned to their home cages and allowed access to water for 1 h.

Conditioning (day 21)

With the water bottle removed, each rat was placed in a Skinner box. Then, 5 min later, each rat received the first of two light foot-shock pairings. House-light parameters were identical to those of the pre-exposure period. The house-light was immediately followed by the footshock (0.5 mA, 1 s). The second light-shock pairing was given 5 min later. After the conditioning period had terminated, animals were returned to their home cages and allowed access to water for 1 h.

Re-baseline day (day 22)

With the water bottle present, each rat was placed in a Skinner box and allowed to drink as in the baseline sessions.

Test day (day 23)

With the water bottle present, each rat was placed in a Skinner box and allowed to drink. When each rat completed 75 licks, the flashing house-light was presented and continued until 5 min had elapsed from stimulus onset. Time bins of 30 s duration commenced from the time of stimulus presentation and the number of licks made by each rat within every time bin was recorded. This measure allowed the pattern of drinking over the course of stimulus presentation to be shown. The amount of suppression of licking for each rat was assessed using a suppression ratio calculated from the time (in seconds) to complete licks 51–75 (pre-stimulus) divided by the time (in seconds) to complete licks 51–100 (pre-stimulus + stimulus on). A suppression ratio of 0.01 indicates total suppression of licking (no latent inhibition), while a ratio of 0.5 indicates no change in licking rate from the pre-stimulus period to the stimulus-on period (latent inhibition).

DRUG Treatment

Test drugs or vehicle were administered by subcutaneous injection in various doses 30 min prior to pre-exposure and conditioning.

EVALUATION

Times to complete licks and the suppression ratios were analyzed independently using a 2 × 6 ANOVA with main factors of pre-exposure and drugs.

MODIFICATIONS OF THE METHOD

Lehmann et al. (1998) studied the long-term effects of repeated maternal separation on three different latent inhibition paradigms.

Pouzet et al. (2004) reported that latent inhibition is spared by NMDA-induced ventral hippocampal lesions, but is attenuated following local activation of the ventral hippocampus by intracerebral NMDA infusion.

Bethus et al. (2005) examined the effects of prenatal stress and gender in latent inhibition.

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E.5.3

Tests Based on the Mechanism of Action

E.5.3.1

Amphetamine Group Toxicity

PURPOSE AND RATIONALE

It is well known that aggregation of mice in small cages greatly enhances the toxicity of amphetamine. The death rate can be reduced by pretreatment with neuroleptics. This phenomenon is generally accepted as an indicator of neuroleptic activity. The increased toxicity results from increased behavioral activation due to aggregation inducing an increase of circulating catecholamines. The mechanism can be understood by the fact that amphetamine is an indirectly acting sympathomimetic amine that exerts its effects primarily by releasing norepinephrine from storage sites in the sympathetic nerves. After administration of high doses of amphetamine, mice exhibit an elevated motor activity which is highly increased by aggregation. This increased behavioral activation is followed by death within 24 h in 80–100% of control animals. Neuroleptics reduce this death rate. In contrast, non-neuroleptic sympatholytics and psychosedative agents like the barbiturates do not produce a dose-related protection. Moreover, anxiolytic agents like benzodiazepines are also found to be ineffective in the prevention of amphetamine group toxicity.

PROCEDURE

Ten male mice of the NMRI-strain are used for each group. They are dosed with the test compound or the standard either orally or intraperitoneally and all placed in glass jars of 18 cm diameter. Untreated animals serve as controls. The test has to be performed at room temperature of 24°C. Thirty min after i.p. or 1 h after oral administration the mice receive 20 mg/kg d-amphetamine subcutaneously. The mortality is assessed 1, 4 and 24 h after dosing.

EVALUATION

The mortality of amphetamine only treated animals is at least 80%. If less than 80% die due to low ambient temperature the test has to be repeated. The estimation of ED₅₀ values for protection and their confidence limits are calculated by probit analysis of the data using the number of dosed vs. the number of surviving animals. Doses of 10 mg/kg chlorpromazine p.o. and 1 mg/kg haloperidol have been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The amphetamine group toxicity test has been used by many investigators and has been found to be a reliable method for detecting neuroleptic activity.

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E.5.3.2**Inhibition of Amphetamine Stereotypy in Rats****PURPOSE AND RATIONALE**

Amphetamine is an indirect acting sympathomimetic agent which releases catecholamines from its neuronal storage pools. In rats the drug induces a characteristic stereotypic behavior. This behavior can be prevented by neuroleptic agents.

PROCEDURE

Groups of 6 Wistar rats with a body weight between 120 and 200 g are used. They are injected simultaneously with d-amphetamine (10 mg/kg s.c.) and the test compound intraperitoneally and then placed individually in stainless-steel cages (40 × 20 × 18 cm). The control groups receive d-amphetamine and vehicle. Stereotypic behavior is characterized by continuous sniffing, licking or chewing and compulsive gnawing. The animals are observed 60 min after drug administration. An animal is considered to be protected, if the stereotypic behavior is reduced or abolished.

EVALUATION

The percent effectiveness of a drug is determined by the number of animals protected in each group. A dose response is obtained by using 10 animals per group at various doses. ED_{50} values can be calculated. The standard neuroleptic drugs have the following ED_{50} values: chlorpromazine 1.75 mg/kg i.p. and haloperidol 0.2 mg/kg i.p.

CRITICAL ASSESSMENT OF THE METHOD

Inhibition of amphetamine-induced stereotypies in rats can be regarded as a simple method to detect neuroleptic activity. However, this may reflect the effects in the corpus striatum which are thought to be responsible for the Parkinsonism-like side effects of neuroleptics.

MODIFICATIONS OF THE METHOD

Ljungberg and Ungerstedt (1985) described a rapid and simple behavioral screening method for simultaneous assessment of limbic and striatal blocking effects of neuroleptic drugs. A low dose of 2 mg/kg d-amphetamine i.p. induces both increased locomotion, thought to reflect an increased dopamine transmission in the nucleus accumbens, and weak stereotypies, thought to reflect an increased dopamine transmission in the neostriatum. The behavior is measured in a combined open field apparatus with holes on the bottom to measure nose-poking and registration of time spent in the corners. Neuroleptics with less propensity to induce unwanted extrapyramidal side effects can be differentiated from classical drugs with more extrapyramidal adverse reactions.

Segal and Kuczenski (1997) described an escalating dose “binge” model of amphetamine psychosis. Rats were exposed to escalating doses of amphetamine (1.0–8.0 mg/kg) before multiple daily injections of relatively high doses of the drug (8 mg/kg every 2 h × 4 injections).

Atkins et al. (2001) described stereotypic behaviors in mice selectively bred for high and low methamphetamine-induced stereotypic chewing.

Machiyama (1992) recommended chronic methylamphetamine intoxication in **Japanese monkeys** (*Macaca fuscata*) as a model of schizophrenia in animals.

Ellenbroek (1991) described the ethological analysis of **Java monkeys** (*Macaca fascicularis*) in a social setting as an animal model for schizophrenia.

Sams-Dodd and Newman (1997) described the effects of the administration regime on the psychotomimetic properties of d-amphetamine in the Squirrel monkey (*Saimiri sciureus*).

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E.5.3.3

Inhibition of Apomorphine Climbing in Mice

PURPOSE AND RATIONALE

Administration of apomorphine to mice results in a peculiar climbing behavior characterized initially by rearing and then full-climbing activity, predominantly mediated by the mesolimbic dopamine system (Costall et al. 1978). The ability of a drug to antagonize apomorphine-induced climbing behavior in the mouse has been correlated with neuroleptic potential (Protais et al. 1976; Costall et al. 1978).

PROCEDURE

Groups of 10 male mice (20–22 g) are treated i.p. or orally with the test substance or the vehicle and placed individually in wire-mesh stick cages. Thirty min afterwards, they are injected s.c. with 3 mg/kg apomorphine. Ten, 20 and 30 min after apomorphine administration, they are observed for climbing behavior and scored as follows:

- 0 = four paws on the floor,
- 1 = fore feet holding the vertical bars,
- 2 = four feet holding the bars.

EVALUATION

The average values of the drug-treated animals are compared with those of the controls, the decrease is expressed as percent. The ED_{50} -values and confidence limits are calculated by probit analysis. Three dose levels are used for each compound and the standard with a minimum of 10 animals per dose level.

CRITICAL ASSESSMENT OF THE TEST

Similar to the enhancement of compulsive gnawing of mice after apomorphine by antidepressant drugs, the suppression of climbing behavior of mice after apomorphine can be used for testing neuroleptic drugs. The test has been modified by various authors.

In contrast to other strains of mice, apomorphine climbing is not induced in DBA2 mice unless sub-

chronic manipulations of brain dopamine transmission are performed (Duterte-Boucher and Costentin 1989).

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E.5.3.4

Inhibition of Apomorphine Stereotypy in Rats

PURPOSE AND RATIONALE

Apomorphine induces a stereotyped behavior in rats, characterized by licking, sniffing and gnawing in a repetitive, compulsive manner, which is an indication of striatal dopaminergic stimulation (Anden et al. 1967; Ernst 1967; Costall and Naylor 1973). Compounds which prevent apomorphine-induced stereotypy antagonize dopamine receptors in the nigrostriatal system (Ljungberg and Ungerstedt 1978; Tarsy

and Baldessarini 1974). Furthermore, antagonism of this behavior is predictive of propensity for the development of extrapyramidal side effects and tardive dyskinesias (Klawans and Rubovits 1972; Tarsy and Baldessarini 1974; Christensen et al. 1976; Clow et al. 1980).

PROCEDURE

For screening, groups of 6 male Wistar rats with a body weight between 120 and 200 g are used. The test drug or the standard are administered i.p. 60 min prior apomorphine dosage. Apomorphine HCl is injected s.c. at a dose of 1.5 mg/kg. The animals are placed in individual plastic cages. A 10 s observation period is used to measure the presence of stereotypic activity such as sniffing, licking and chewing 10 min after apomorphine administration. An animal is considered protected if this behavior is reduced or abolished.

EVALUATION

The percent effectiveness of a drug is determined by the number of animals protected in each group. With a group size of 10 animals dose response curves are obtained and ED_{50} values calculated. ED_{50} values were found to be 0.2 mg/kg s.c. for haloperidol and 5.0 mg/kg for chlorpromazine, whereas clozapine was ineffective even at high doses.

MODIFICATIONS OF THE METHODS

Puech et al. (1978) studied the effects of several neuroleptic drugs on hyperactivity induced by a low dose of apomorphine in mice.

Apomorphine induces stereotypic behavior in a variety of species including pigeons. The symptoms in pigeons are manifested as pecking against the wall of the cage or on the floor. Aksas et al. (1984) described a method registering the pecking after apomorphine by a microphone, amplification through a pulse preamplifier and registration with a polygraph. The effect of apomorphine was dose-dependent decreased by yohimbine and neuroleptics.

Stereotyped behavior in guinea pigs induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

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E.5.3.5

Yawning/Penile Erection Syndrome in Rats

PURPOSE AND RATIONALE

Yawning is a phylogenetically old, stereotyped event that occurs alone or associated with stretching and/or penile erection in humans and in animals from reptiles to birds and mammals under different conditions (Argiolas and Melis 1998). The yawning-penile erection syndrome can be induced in rats by apomorphine and other dopamine autoreceptor stimulants (Ståhle

and Ungerstedt 1983; Gower et al. 1984) and can be antagonized by haloperidol and other dopamine antagonists. Antagonism against this syndrome can be regarded as indication of antipsychotic activity (Furukawa 1996).

Besides the **dopaminergic** system in this behavior (Mogilnicka and Klimek 1977; Baraldi et al. 1979; Benassi-Benelli et al. 1979; Nickolson and Berendsen 1980; Gower et al. 1984, 1986; Dourish et al. 1985; Doherty and Wisler 1994; Kurashima et al. 1995; Bristow et al. 1996; Fujikawa et al. 1996a; Asencio et al. 1999) also the **serotonergic** (Baraldi et al. 1977; Berendsen and Broekkamp 1987; Berendsen et al. 1990; Protais et al. 1995; Millan et al. 1997), the **cholinergic** (Yamada and Furukawa 1980; Fujikawa et al. 1996b), the **GABAergic** (Zarrindast et al. 1995), the **NO system** (Melis et al. 1995, 1996, 1997a, b), and **steroid** as well as **peptide hormones** (Bertolini and Baldari 1975; Bertolini et al. 1978; Holmgren et al. 1980; Berendsen and Nickolson 1981; Berendsen and Gower 1986; Gully et al. 1995) are involved (Argiolas and Melis 1998).

PROCEDURE

Naive male Wistar rats, weighing 220–280 g, are housed under controlled 12 h light-dark cycle with free access to standard food pellets and tap water. Rats are pretreated with subcutaneous injection of the antagonist 30 min prior to injections of the agonist, such as apomorphine (0.02 to 0.25 mg/kg s.c.) or physostigmine (0.02 to 0.3 mg/kg s.c. or i.p.). After administration of the agonist, rats are placed in individual transparent Perspex cages. A mirror is placed behind the row of observation cages to facilitate observation of the animals for penile erections and yawns. Yawning is a fixed innate motor pattern characterized by a slow, wide opening of the mouth. A penile erection is considered to occur when the following behaviours are present: repeated pelvic thrusts immediately followed by an upright position, an emerging, engorged penis which the rats proceeds to lick while eating the ejaculate. The number of penile erections and yawns is counted for 30 min following the last injection.

EVALUATION

The results are expressed as the mean number of yawns and of penile erections per group \pm SEM. The statistical significance is determined by comparing the results of each group with the results of the relevant control group using a non-parametric rank sum test.

CRITICAL ASSESSMENT OF THE METHOD

Ferrari et al. (1993) published some evidence that yawning and penile erection in rats underlie different neurochemical mechanisms. Nevertheless, the procedure can be regarded as an useful behavioral tool to study putative antipsychotic activity of new compounds.

MODIFICATIONS OF THE METHOD

Two sublines of Sprague Dawley rats were bred for high- and low yawning frequency in males (Eguibar and Moyaho 1997).

Apomorphine produced more yawning in Sprague Dawley rats than in F344 rats (Tang and Himes 1995).

Sato-Suzuki et al. (1998) evoked yawning by electrical or chemical stimulation in the paraventricular nucleus of anesthetized rats.

The yawning – penile erection syndrome in rats can be elicited by injections of 50 ng NMDA or AMPA (Melis et al. 1994, 1997b) into the paraventricular nucleus of the hypothalamus or intracerebroventricular injection of 50 ng oxytocin (Melis et al. 1997a) or ACTH (Genedani et al. 1994; Poggioli et al. 1998) or α -MSH (Vergoni et al. 1998).

Champion et al. (1997), Bivalacqua et al. (1998) studied the effect of intracavernosal injections of adrenomedullin and other peptide hormones on penile erections in **cats**.

Dopaminergic influences on male sexual behavior of **rhesus monkeys** were studied by Pomerantz (1990, 1992).

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E.5.3.6**Inhibition of Mouse Jumping****PURPOSE AND RATIONALE**

Lal et al. (1975) described a jumping response in mice after administration of L-dopa in amphetamine pretreated animals where the number of jumps can be objectively counted. The mouse jumping is due to dopaminergic overstimulation similar to that seen in rats when stereotypy is induced by higher doses of amphetamine. The phenomenon can be blocked by neuroleptics.

PROCEDURE

Male CD-1 mice weighing 22–25 g are injected with 4 mg/kg d-amphetamine sulfate, followed 15 min later by an i.p. injection of 400 mg/kg L-dopa. The mice spontaneously begin to jump at a high rate. A median of 175 jumps can be observed in these mice during 60 min. Since mice do not show any jumping after saline administration, the responses after drug administration are specific and can be measured automatically through a pressure-sensitive switch closure or properly positioned photoelectric beam disruptions. Test compounds are administered 60 min prior to L-dopa injection.

EVALUATION

Jumps of mice treated with test drugs or standard are counted and expressed as percentage of jumps in amphetamine/L-dopa treated animals. Using various doses, ED_{50} values with 95% confidence limits are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method has been found to be sensitive and rather specific for neuroleptic drugs.

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E.5.3.7**Antagonism Against MK-801 Induced Behavior****PURPOSE AND RATIONALE**

Dizocilpine (MK-801), a non-competitive NMDA antagonist, induces a characteristic behavior in rat and mice, which is regarded as a model of psychosis (Andiné et al. 1999). In mice MK-801 induces a characteristic stereotypy marked by locomotion and falling behavior through both dopamine dependent and dopamine independent mechanisms (Carlson and Carlson 1989; Verma and Kulkarni 1992). Antipsychotic agents dose-dependent antagonize this MK-801 induced behavior.

PROCEDURE

Male CD-1 mice (20–30 g) are individually placed in activity boxes lined with wire mesh flooring and allowed to acclimate for 60 min. The animals are then dosed with compounds 30 min prior to subcutaneous administration of MK-801 at 0.2 mg/kg. The mice are observed for locomotion and the presence of falling behavior 15 min following MK-801 administration.

EVALUATION

ED_{50} values and 95% confidence limits are calculated by the Litchfield and Wilcoxon method.

MODIFICATIONS OF THE METHOD

Deutsch and Hitri (1993), Rosse et al. (1995), Deutsch et al. (2002, 2003), and Mastropaolo et al. (2004) described methods to measure the MK-801-induced explosive behavior in mice, called “popping”.

Farber et al. (1996) showed that neuroleptic drugs can prevent neuronal vacuolization and necrosis induced by MK-801 (Fix et al. 1993).

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E.5.3.8

Phencyclidine Model of Psychosis

PURPOSE AND RATIONALE

Phencyclidine- (PCP-) induced symptoms in rats are considered as a model of psychosis (Ogawa et al. 1994; Halberstadt 1995; Steinpreis 1996; Abi-Saab et al. 1998; Sams-Dodd 1998a; Jentsch and Roth 1999; Phillips et al. 2000; Farber 2003; Morris et al. 2005).

PCP-induced symptoms can be antagonized by neuroleptic drugs (Witkin et al. 1997; Sams-Dodd 1998b; Javitt et al. 2004).

Cartmell et al. (1999) found that metabotropic glutamate receptor agonists selectively attenuate phencyclidine versus d-amphetamine motor behaviors in rats.

PROCEDURE

Behavior of male Sprague Dawley rats weighing 250–300 g was monitored while in transparent, plastic shoebox cages of the dimensions 45 × 25 × 20 cm, with 1 cm depth of wood clips as bedding, and a metal grill on the top of the cage. Motor monitors consisted of a rectangular rack of 12 photobeams arranged in a 8 × 4 formation. Shoe boxes were placed inside these racks, enabling the activity of the rat to be monitored in a home-cage environment. The lower rack was positioned at a height of 5 cm, which allowed the detection of PCP-induced head movements in addition to movements of the body of the rat. Rearing activity was detected by a second rack placed 10 cm above the first. Rats were placed in the cage for an acclimation period of 30 min, then removed, administered the

test compounds s.c. or sterile water and then returned to the same cages. After 30 min, the rats were given an s.c. injection of PCP or amphetamine or sterile water and once again returned to the cages. Motor activity was monitored for the following 60 min resulting in the measurement of three different parameters: ambulations (pattern of beam breaks indicating that the animal had relocated its entire body), fine movements (non-ambulatory beam breaks), and time at rest. An indication of rearing activity was detected in the upper rack of photobeams.

EVALUATION

Data were analyzed by a one-way ANOVA, and then post-hoc comparisons for each dose group versus control or PCP alone or PCP and test compound were made using Newman-Keuls Multiple Comparison test.

MODIFICATIONS OF THE METHOD

Furuya et al. (1998) investigated the effects of a strychnine-insensitive glycine site antagonist on the hyperactivity and the disruption of prepulse inhibition induced by phencyclidine (PCP) in rats.

Redmond et al. (1999) tested the effects of acute and chronic antidepressant administration on PCP-induced locomotor hyperactivity.

Boulay et al. (2004) tested a putative atypical antipsychotic for improvement of social interaction deficits induced by PCP in rats.

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E.5.3.9

Inhibition of Apomorphine-Induced Emesis in the Dog

PURPOSE AND RATIONALE

The blockade of centrally acting dopaminergic mechanisms is considered to play a major role in suppression of psychotic reactions in schizophrenia. Apomorphine, regarded as a direct dopaminergic agonist, produces a pronounced emetic effect in dogs and the blockade of apomorphine emesis is used as an indication of dopaminergic blockade. However, although both antiemetic activity and antipsychotic activity are thought to be due to dopaminergic blockade, the sites of action are in different brain areas and there is a lack of complete correlation of these activities.

PROCEDURE

Adult beagle dogs of either sex are used in treatment groups of three to nine dogs/dose. The dogs are given the test compounds in a gelatin capsule; they are then dosed with 0.15 mg/kg apomorphine s.c. at various intervals after administration of the test compound. The dogs are first observed for overt behavioral effects, e. g., pupillary response to light, changes in salivation, sedation, tremors, etc; then, after the administration

of apomorphine, the dogs are observed for stereotypic sniffing, gnawing and the emetic response. Emesis is defined as wrenching movements followed by an opening of the mouth and either attempted or successful ejection of stomach content.

EVALUATION

If the experimental compound is anti-emetic in the primary screen, the dose is progressively lowered to obtain a minimal effective dose or an ED_{50} value. The ED_{50} values for haloperidol and chlorpromazine were found to be 0.06 mg/kg p.o. and 2.0 mg/kg p.o., respectively. Clozapine was not effective at doses between 2 and 10 mg/kg. p.o.

CRITICAL ASSESSMENT OF THE METHOD

The method has been extensively used by several laboratories. However, since non-classical neuroleptics like clozapine did not show pronounced activity the test has been abandoned. Moreover, tests in higher animals like dogs are limited due to regional regulations.

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E.5.3.10

Purposeless Chewing in Rats

PURPOSE AND RATIONALE

Purposeless chewing can be induced in rats by directly acting cholinergic drugs or cholinesterase inhibitors (Rupniak et al. 1983), which can be blocked by anti-muscarinic agents. The chewing behavior has been proposed to be mediated through central M_2 receptors rather than via central M_1 sites (Stewart et al. 1989). Chewing can also be induced by chronic administration of neuroleptics in rats (Clow et al. 1979; Iversen et al. 1980). Purposeless chewing is mediated by dopaminergic and nicotinic mechanisms.

PROCEDURE

Male albino rats are housed 10 per cage at room temperature and kept on a 12 h light-dark cycle. For the experiments, rats are placed individually in a large glass cylinder (height 30 cm, diameter 20 cm) at $21 \pm 1^\circ\text{C}$ and allowed to habituate for 15 min before injection of drugs. The antagonists, e.g. sulphiride or mecamlamine as standards, are given at different doses 30 min before treatment either with 0.01 mg/kg nicotine or 1 mg/kg pilocarpine i.p. Number of chewings are counted by direct observation immediately after drug administration. The results are presented as number of chews in a 30 min period.

EVALUATION

Analysis of variance (ANOVA), followed by Newman-Keuls tests, are used to evaluate the significance of the results obtained. $P < 0.05$ is considered as significant.

MODIFICATIONS OF THE METHOD

Tremulous jaw movements induced by tacrine (Cousins et al. 1999) can be antagonized by antipsychotic drugs (Betz et al. 2005; Ishiwarii et al. 2005).

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E.5.3.11**Models of Tardive Dyskinesia****PURPOSE AND RATIONALE**

Tardive dyskinesia is a severe side-effect of traditional neuroleptics affecting a considerable number of patients probably based on a genetic disposition, being characterized by involuntary movements of the oral region. Various authors used rats as animal model for tardive dyskinesia, either after treatment with reserpine (Waddington et al. 1990; Neisewander 1994; Bergamo et al. 1997; Quieroz and Frussa-Filho 1999; Andressen 2000; Casey 2000; Van Kampen et al. 2000; Calvente et al. 2002; Abílio et al. 2003; Peixoto et al. 2003) or haloperidol (Takeuchi et al. 1998; Harvey and Nel 2003; Naidu et al. 2003; Burger et al. 2005). Several authors compared the effects of different neuroleptics (See and Ellison 1990; Tamminga et al. 1994) or studied potential antagonistic effects (Takeuchi et al. 1998; Quieroz and Frussa-Filho 1999; Abílio et al. 2003; Naidu et al. 2003; Peixoto et al. 2003).

Burger et al. (2005) found that ebselen attenuates haloperidol-induced orofacial dyskinesia and oxidative stress in rat brain.

PROCEDURE

Male Wistar rats weighing 270–320 g were injected sc. once a week with 12 mg/kg haloperidol decanoate for 4 weeks. Another group was pretreated with 30 mg/kg ebselen and received in addition to haloperidol every other day an ip. injection of 30 mg/kg ebselen.

The animals were observed for the quantification of orofacial dyskinesia just before haloperidol administration and on the 7th, 14th, 21st and 28th day after the first administration of haloperidol.

Rats were placed individually in cages (20 × 20 × 19 cm) containing mirrors under the floor and behind the back wall of the cage to allow behavioral quantification when the animal was faced away from the observer. To quantify the occurrence of oral dyskinesia, the incidence of tongue protrusions, vacuous chewing movements frequency, and the duration of facial twitching were recorded for 15 min. Observers were blind to drug treatment.

EVALUATION

Data were analyzed by a three-way ANOVA, followed, when appropriate, by univariate analysis and Duncan's Post Hoc test.

MODIFICATIONS OF THE METHOD

Several authors used monkeys (*Cebus apella* or *Macaca speciosa*) to evaluate the effect of neuroleptics to induce tardive dyskinesia-like symptoms (Gunne and Barany 1979; Domino 1985; Werge et al. 2003).

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E.5.3.12

Single Unit Recording of A9 and A10 Midbrain Dopaminergic Neurons

PURPOSE AND RATIONALE

Interactions with central nervous system dopamine pathways are crucial for the expression of antipsychotic effects seen with clinically effective neuroleptics. These interactions also have a role in the expression of several of the neurological side effects seen with these agents. Extracellular single unit recording techniques of rat A9 (substantia nigra) and A10 (ventral tegmental area) dopamine neurons show that after acute treatment with neuroleptics the number of spontaneously firing cells is increased in both areas. After repeated treatment (21 days) a decrease was found with all neuroleptics in the A10 neurons, whereas in the A9 cell only compounds with clinically evident extrapyramidal side effects induced a decrease. Clozapine which is believed not to produce extrapyramidal side effects resulted in the depolarization inactivation of A10 neurons but not A9 cells. The method provides a prediction of a compound's anti-psychotic potential as well as potential neurological side effects (Chiodo and Bunney 1983).

PROCEDURE

Male Wistar rats weighing 280–360 g are anesthetized with chloralhydrate intraperitoneally. The animal is mounted in a stereotaxic apparatus (Kopf, model 900). The cranium is exposed, cleaned of connective tissue and dried. The skull overlying both the substantia nigra (A9: anterior (A) 3000–3400 μm, lateral (L) 1800–2400 μm from lambda), and the ventral tegmental area (A10: A 3000–3400 μm, L 400–1000 μm from lambda) (Paxinos and Watson 1986) is removed. Using the dura as point of reference, a micropipette driven by a hydraulic microdrive is lowered through the opening of the skull at vertical 6000–8500 μm. Spontaneously firing dopamine neurons within both the substantia ni-

gra and the ventral tegmental area are counted by lowering the electrode into twelve separate tracks (each track separated from the other by 200 μm) in each region. The sequence of these tracks is kept constant, forming a block of tissue which can be reproducibly located from animal to animal.

Extracellular neuronal signals are sampled using a single barrel micropipette approximately one μm at its tip, and filled with 2 M NaCl saturated with 1% pontamine sky blue dye (*in vitro* impedance between 5 and 10 M Ω). Electrical potentials are passed through a high-impedance preamplifier and the signal is sent to a window discriminator which converts potentials above background noise levels to discrete pulses of fixed amplitude and duration. Only cell whose electrophysiological characteristics match those previously established for midbrain dopamine neurons are counted. In an anesthetized rat, a neuron is considered to be dopaminergic if it displays a triphasic positive-negative-positive spike profile of 0.4 to 1.5 mV amplitude and 2.5 ms duration, firing in an irregular pattern of 3 to 9 Hz with occasional bursts characterized by progressively decreasing spike amplitude and increasing spike duration.

At the end of each experiment, the location of the last recorded track tip is marked by passing 25 microampere cathodal current through the recording micropipet barrel for 15 min in order to deposit a spot of dye. The rat is sacrificed, the brain is then removed, dissected and frozen on a bed of dry ice. Frozen serial sections (20 μm in width) are cut, mounted and stained with cresyl violet and examined using a light microscope.

Animals pretreated with vehicle prior to neuronal sampling serve as controls. For animals that are used in an acute single-unit dopamine neuron sampling assay, test compounds are administered intraperitoneally one hour prior to the beginning of dopamine neuron sampling. For animals used in a chronic single-unit dopamine sampling assay, the compounds are administered once a day for 21 days, and dopamine neuron sampling is begun 2 h after the last dose on the 21st day.

EVALUATION

Drug treatment groups are compared to vehicle groups with a one-way ANOVA with a post hoc Neuman-Keuls analysis for significance.

MODIFICATIONS OF THE METHOD

Nybäck et al. (1975) tested the influence of tricyclic antidepressants on the spontaneous activity of nore-

pinephrine-containing cells of the locus caeruleus in anesthetized rats.

Scuvée-Moreau and Dreesse (1979) studied the effect of various antidepressant drugs on the firing rate of locus caeruleus and dorsal raphe neurons of the anesthetized rat with extracellular microelectrodes.

Using the method of single-unit recording of spontaneous firing of locus caeruleus neurons in rats, Cedarbaum and Aghajanian (1977) studied the inhibition by micro-iontophoretic application of catecholaminergic agonists.

Marwaha and Aghajanian (1982) examined in single unit studies the actions of adrenoceptor antagonists at *alpha*-1 adrenoceptors of the dorsal raphe nucleus and the dorsal lateral geniculate nucleus and *alpha*-2 adrenoceptors of the nucleus locus caeruleus.

Mooney et al. (1990) studied the organization and actions of the noradrenergic input to the superior colliculus of the hamster using micro-iontophoretic techniques together with extracellular single unit recording.

Bernardini et al. (1991) studied *in vitro* with brain slices of mice the amphetamine-induced and spontaneous release of dopamine from A9 and A10 cell dendrites.

Santucci et al. (1997) investigated the effects of synthetic neurotensin receptor antagonists on spontaneously active A9 and A10 neurones in rats.

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E.5.3.13

In Vivo Voltammetry

PURPOSE AND RATIONALE

Various groups (Lane et al. 1979, 1987, 1988; Blaha and Lane 1983, 1984, 1987; Crespi et al. 1984; Marsden et al. 1984; Maidment and Marsden 1987a, b; Armstrong-James and Millar 1979, 1984; Kawagoe et al. 1993) described *in vivo* voltammetry as an electrochemical technique that uses carbon fiber microelectrodes stereotactically implanted in brain areas to monitor monoamine metabolism and release. De Simoni et al. (1990) reported on a miniaturized optoelectronic system for telemetry of *in vivo* voltammetric signals in freely moving animals.

PROCEDURE

Carbon fibre working electrodes are made from pyrolytic carbon fibres supported in a pulled glass capillary (Armstrong-James and Millard 1979; Sharp et al. 1984) and electrically pretreated for simultaneous recording of ascorbic acid DOPAC and 5-HIAA (Crespi et al. 1984).

Male Sprague Dawley rats weighing 270–340 g are anesthetized with a 2–3% halothane O₂/NO₂ mixture (1:1) and held in a stereotaxic frame. Reference and auxiliary electrodes are positioned on the surface of the dura through 1 mm holes drilled in the cra-

nium and held in place with dental cement. Holes, approx. 2 mm in diameter, are drilled in the cranium above the left or right nucleus accumbens and contralateral anterior striatum, and the underlying dura is broken with a hypodermic needle. A working electrode is lowered in one of the above regions and cemented in place. A second electrode is then implanted in the remaining structure. The coordinates, measured from the bregma, are as follows: nucleus accumbens–rostr-caudal +3.4 mm; medio-lateral ± 1.4 mm; dorso-ventral –7 mm; striatum–rostr-caudal +2.8 mm; medio-lateral ± 2.6 mm; dorso-ventral –5.5 mm.

Drugs are injected subcutaneously. Voltammograms are recorded using a Princeton Applied Research 174A polarographic analyzer alternatively from each region every 5 min and after a 1 h stabilization period.

EVALUATION

Voltammetric data are expressed as percentage changes from pre-injection control values using the mean of the last 6 peak heights before administration of drug as the 100% value. However, statistical analysis of the data is carried out on the absolute peak heights using a paired Student's *t*-test to compare 6 pre-injection control peak heights with those after administration of drug at selected time points.

MODIFICATIONS OF THE METHOD

Swiergiel et al. (1997) constructed voltammetric probes from stainless steel and fused silica tubing sheathing carbon fibers and compared them with commercially available glass-sealed IVEV-5 electrodes. This type of electrodes can be easily manufactured and does not require any special equipment.

Parada et al. (1994, 1995) described a triple-channel swivel suitable for intracranial fluid delivery and microdialysis experiments which can be equipped with three electrical channels for *in vivo* voltammetry and measurement of intracranial temperature with a thermocouple.

Frazer and Daws (1998) used electrodes coated with a perfluorinated ion exchange resin (Nafion) to assess serotonin transporter function *in vivo* by **chrono-amperometry** whereby voltage is applied to the electrode in a pulsed manner and the current obtained measured as a function of time.

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E.5.4

Genetic Models of Psychosis

E.5.4.1

The Heterozygous Reeler Mouse

PURPOSE AND RATIONALE

Reelin is an extracellular matrix protein secreted by GABAergic interneurons that, acting through pyramidal neuron integrin receptors, provides a signal for dendritic spine plasticity. The gene responsible for a mouse mutant strain is called *reeler* (D'Arcangelo and Curran 1998; Lombroso and Goldowitz 1998; Fatemi 2001; Pappas et al. 2003). Heterozygous *reeler* mice that exhibit a 50% downregulation of reelin expression replicate the dendritic spine and GABAergic defects described in human schizophrenia (Larson et al. 2003). The heterozygote *reeler* mouse was recommended as a model for the development of a new generation of antipsychotics (Tueting et al. 1999; Rowley et al. 2001; Costa et al. 2002). This view has been challenged by Podhorna and Didriksen (2004).

Tomasiewicz et al. (1993) and Wood et al. (1998) proposed **NCAM-180 knockout mice** with a deletion of the neural cell adhesion molecule variant (N-CAM-180) displaying increased lateral ventricle size and a reduced prepulse inhibition of startle response as model for schizophrenia.

Dirks et al. (2003) reported reversal of startle gating deficits in **transgenic mice overexpressing corticotropin-releasing factor** by antipsychotic drugs.

Van den Buuse (2003) showed deficient prepulse inhibition of acoustic startle in **Hooded-Wistar** rats compared with Sprague Dawley rats suggesting that the Hooded-Wistar line could be a useful genetic animal model to study the interaction of glutaminergic and dopaminergic mechanisms in anxiety and schizophrenia.

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E.5.4.2

The Hooded-Wistar Rat

PURPOSE AND RATIONALE

Van den Buuse (2003), Lodge et al. (2003), and Martin et al. (2004) suggested that the Hooded-Wistar line (fawn-hooded rats) could be a useful genetic animal model to study the interaction of glutamatergic and dopaminergic mechanisms in anxiety and schizophrenia.

Broderick (2002) compared hippocampal serotonin and norepinephrine release during open-field behavior in Sprague Dawley animals with the Fawn-Hooded animals model of depression.

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E.6 Antidepressant Activity

E.6.0.1

General Considerations

The first antidepressant drugs were detected by serendipity in clinical trials. Iproniazid was developed for the treatment of tuberculosis. The observation of mood-elevating effects was followed by the detection of the inhibition of the enzyme monoamine oxidase. During clinical investigation of phenothiazine analogs as neuroleptics, imipramine was found to be relatively ineffective in agitated psychotic patients but showed remarkable benefit in depressed patients. Later on, inhibition of uptake of biogenic amines was found to be the main mechanism of action resulting in downregulation of β -receptors (Vetulani et al. 1976). Influence on α_2 -adrenoreceptors (Johnson et al. 1980) was discussed as well. Several lines of preclinical and clinical evidence indicate that an enhancement of 5-HT-mediated neurotransmission might underlie the therapeutic effect of most antidepressant treatments (Blier and de Montigny 1994).

Animal models of depression have been reviewed by Porsolt et al. (1991), Panksepp et al. (1991), Willner and Muscat (1991), and Cryan et al. (2002).

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E.6.1

In Vitro Methods

E.6.1.1

Inhibition of [3 H]-Norepinephrine Uptake in Rat Brain Synaptosomes

PURPOSE AND RATIONALE

As shown by Hertting and Axelrod (1961) the neuronal re-uptake mechanism for norepinephrine is the most important physiological process for removing and inactivating norepinephrine in the synaptic cleft. This uptake is inhibited by cocaine, certain phenylethylamines and antidepressants. This mechanism is considered as one of the most important modes of action of anti-depressants leading to receptor down-regulation. In the brain, the hypothalamus shows the highest level and greatest uptake of noradrenaline. Therefore, this region is used for testing potential antidepressant drugs.

PROCEDURE

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. The hypothalamic region is prepared, weighed, and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvehjem homogenizer. The homogenate is centrifuged at 1000 g at 0–4°C for 10 min. The supernatant is decanted and used for the uptake experiments.

Assay

200 μ l of tissue suspension are incubated with 800 μ l 62.5 nM 3 H-norepinephrine in Krebs-Henseleit bicarbonate buffer and 20 μ l of the appropriate drug concentration (or the vehicle) at 37°C under a 95% O₂/5% CO₂ atmosphere for 5 min. For each assay, 3 tubes are incubated with 20 μ l of vehicle at 0°C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g for 10 min. The supernatant fluid is aspirated and the pellets dissolved adding 1 ml of solubilizer (Triton X-100 + 50% ethanol, 1:4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation cock-

tail. Active uptake is the difference between cpm at 37°C and 0°C.

EVALUATION

The percent inhibition at each drug concentration is the mean of 3 determinations. IC_{50} values are derived from log-probit analysis. IC_{50} values for the standard drugs desipramine and nortriptyline are around 20 nM.

MODIFICATIONS OF THE METHOD

Pacholczyk et al. (1991) described the expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter.

Tejani-Butt (1992) recommended [3H]nisoxetine as radioligand for quantitation of norepinephrine uptake sites.

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E.6.1.2

Inhibition of [3H]-Dopamine Uptake in Rat Striatal Synaptosomes

PURPOSE AND RATIONALE

High affinity, saturable, temperature and sodium-dependent transport of 3H -dopamine has been observed in various tissue preparations from different brain regions. The area striata has a high content of dopamine and is suitable for uptake experiments. The 3H -dopamine uptake is inhibited by cocaine, certain

phenylethylamines and antidepressants like nomifensine and bupropion, but not by tricyclic antidepressants. The test can be used to characterize the mode of action of antidepressant drugs.

PROCEDURE

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Corpora striata are prepared, weighed and homogenized in 9 volumes of ice cold 0.32 M sucrose solution using a Potter-Elvehjem homogenizer. The homogenate is centrifuged at 1000 g at 0–4°C for 10 min. The supernatant is decanted and used for the experiments.

Assay

100 μ l of tissue suspension are mixed with 900 μ l 55.5 nM 3H -dopamine solution in Krebs-Henseleit bicarbonate buffer and 20 μ l of drug solution in appropriate concentration (or the vehicle as control). The tubes are incubated at 37°C under a 95% O_2 /5% CO_2 atmosphere for 5 min. For each assay, 3 tubes are incubated with 20 μ l of vehicle at 0°C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g for 10 min. The supernatant fluid is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X-100 + 50% ethanol, 1:4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml liquid scintillation counting cocktail. Active uptake is the difference between cpm at 37°C and 0°C.

EVALUATION

The percent inhibition at each drug concentration is the mean of 3 determinations. IC_{50} values are derived from log-probit analyses. IC_{50} values for nomifensine are 460 nM, but >20,000 nM for tricyclic antidepressants.

MODIFICATIONS OF THE METHOD

Elsworth et al. (1993) differentiated between cocaine-sensitive and -insensitive dopamine uptake in various brain areas.

Cloning and pharmacological characterization of rat, bovine, and human dopamine transporters have been described (Giros et al. 1991; Kilty et al. 1991; Shimada et al. 1991; Usdin et al. 1991; Giros et al. 1992).

Binding characteristics of the dopamine transporter were studied (Reith et al. 1992; Rothman et al. 1992).

[3H]- 3β -(*p*-fluorophenyl)tropan-2 β -carboxylic acid methyl ester ([3H]WIN 35,428) is used as ligand

for the dopamine transporter (Carroll et al. 1992; Cline et al. 1992).

[³H]GBR12935 (1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine) was used as labeled ligand by Richfield (1991), Nakachi et al. (1995).

Cocaine receptors are specifically labeled with [³H]WIN 35,428 indicating the role of the dopamine transport system in mediating the behavioral effects and the abuse of cocaine (Madras et al. (1989).

Laruelle et al. (1993) reported **single photon emission computed tomography (SPECT)** imaging of dopamine and serotonin transporters in nonhuman primates.

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E.6.1.3

Inhibition of [³H]-Serotonin Uptake

PURPOSE AND RATIONALE

Selective inhibitors of serotonin uptake (SSRIs) became the most powerful and widely used antidepressants (Pacher et al. 2001; Fray et al. 2006). These include citalopram (D'Amato et al. 1987; Brøsen and Narajo 2001), fluoxetine, sertraline, paroxetine (Pacher et al. 2001), and escitalopram (Sánchez et al. 2003; Murdoch and Keam 2005).

These antidepressants block the reuptake of 5-HT. ³H-5-HT transport in brain has been found to be saturable, sodium- and temperature-dependent, to be inhibited by several agents, such as ouabain, tryptamine analogs, and tricyclic antidepressants. Apparently, the 5-HT uptake can be differentiated from catecholamine uptake. Therefore, the test can be used to detect com-

pounds that inhibit serotonin uptake into rat brain synaptosomes and may be potential antidepressants.

PROCEDURE

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Either the whole brain minus cerebellum or the hypothalamus is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvehjem homogenizer. The homogenate is centrifuged at 1000 *g* at 0–4°C for 10 min. The supernatant is decanted and used for further uptake experiments.

Assay

Two hundred μ l of tissue suspension are mixed with 800 μ l 62.5 nM ³H-5-HT solution in Krebs-Henseleit bicarbonate buffer and 20 μ l of drug solution in the appropriate concentration (or the vehicle as control). The tubes are incubated at 37°C under 95% O₂/5% CO₂ atmosphere for 5 min. For each assay, 3 tubes are incubated with 20 μ l of the vehicle at 0°C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 *g* for 10 min. The supernatant is aspirated and the pellets are dissolved by adding 1 ml of solubilizer (Triton X100+50% ethanol, 1+4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation counting cocktail. Active uptake is the difference between cpm at 37°C and 0°C.

EVALUATION

The percent inhibition of each drug concentration is the mean of 3 determinations. *IC*₅₀ values are calculated from log-probit analyses. Standard drugs, such as chlorimipramine show *IC*₅₀ values in the order of 10 nM for ³H-5-HT uptake in rat synaptosomes from hypothalamus.

MODIFICATIONS OF THE METHOD

Similar methods were used by D'Amato et al. (1987), Weinshank et al. (1992), Cheng et al. (1993), Hatanaka et al. (1996), and Tordera et al. (2002).

Sánchez et al. (2003) and Chen et al. (2005) studied [³H]-5-HT uptake inhibition in a COS-1 cell line stably transfected with h5-HTT.

Hallstrom et al. (1976) studied the platelet uptake of 5-hydroxytryptamine and dopamine in patients with depression.

Cloning of functional serotonin transporters has been described by Blakely et al. (1991) and by Hoffman et al. (1991). The role of serotonin in the mode

of action of antidepressant drugs has been discussed by Hyttel and Larsen (1985), Åsberg and Mårtensson (1993), Hyttel (1994), Blier and de Montigny (1997), Keane and Soubrié (1997).

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E.6.1.4

Binding to Monoamine Transporters

PURPOSE AND RATIONALE

There is substantial clinical and experimental evidence that lesions in the serotonergic and noradrenergic systems are responsible for depression and that antidepressant treatment can reverse these alterations (Leonard 2000). Monoamine transporters are principle targets of widely used therapeutic drugs including antidepressants, psychostimulants and the addictive drug cocaine (Madras et al. 1996; Fleckenstein et al. 1999). The termination of neurotransmission is achieved by rapid uptake of the released neurotransmitter by high-affinity neurotransmitter transporters. Most of these transporters are encoded by a family of genes (Na⁺/Cl⁻ transporters) having a similar membrane topography of 12 transmembrane helices. An evolutionary tree revealed five distinct subfamilies: GABA-transporters, monoamine transporters, amino acid transporters, “orphan” transporters and bacterial transporters (Nelson 1998).

Tatsumi et al. (1997) described the pharmacological profile of antidepressants at human monoamine transporters.

PROCEDURE

Expression of Human Transporters

The human serotonin transporter cDNA is directionally ligated into the expression vector pRc/CMV and transfected into HEK293 (human embryonic kidney) cells by the Ca²⁺ method. The human dopamine transporter cDNA is directionally ligated into the expression vector pcDNA3 and transfected into HEK293 cells, also by the Ca²⁺ method.

Cell Culture

The cell lines are grown, passaged, and harvested in 150 mm Petri dishes with 17.5 ml of Dulbecco's modified Eagle's medium containing 0.1 mM nonessential amino acid solution, 5% fetal clonebovine serum product and 1 U/ μ l penicillin and streptomycin solution. They are incubated in 10% CO₂, 90% air at 37°C and 100% humidity. The selecting antibiotic geneticin sulfate (250 μ g/ml) is used continuously for culture of cells expressing the norepinephrine transporter.

Membrane Preparations

For the preparation of the homogenates, the medium is removed by aspiration. The cells are washed with 41 modified Puck's D1 solution (solution 1) (Pfenning and Richelson 1990) and then incubated for 5 min at 37°C in 10 ml solution 1 and 100 mM EGTA. Afterwards cells are removed from the surface by scraping with a rubber spatula, placed in a centrifuge tube, and collected by centrifugation at 110 *g* for 5 min at 4°C. The supernatants are decanted. The pellets are resuspended in the respective binding assay buffer by use of a Polytron (Brinkman Instruments, Westbury, NY) for 10 s at setting 6. The mixture is then centrifuged at 35,600 *g* for 10 min at 4°C. The pellets are suspended in the same volume of the respective buffer and the centrifugation is repeated. The supernatants are decanted and the final pellets are suspended in the respective buffer and stored at -80°C until assayed. The final protein concentration is determined by the Lowry assay using bovine serum albumin as standard.

Radioligand Binding Assays

[³H] imipramine Binding to Human Serotonin Transporter. Radioligand binding assays are performed by a modification of the method of O'Riordan et al. (1990) with a binding buffer containing 50 mM Tris, 120 mM NaCl, and 5 mM KCl (pH 7.4). Compounds to be tested are dissolved in 5 mM HCl and run in duplicate over at least 11 different concentrations against 1 nM [³H]imipramine (specific activity 46.5 Ci/mmol) with 15 μ g/tube membrane protein for 30 min at 22°C. Nonspecific binding is determined in the presence of 1 μ M imipramine. With the use of a 48-well Brandel cell harvester, the assay is terminated by rapid filtration through a GF/B filter presoaked with 0.2% polyethylenimine. The filter strips are rinsed five times with ice-cold 0.9% NaCl. Finally, each filter is placed in a scintillation vial containing 6.5 ml of Redi-Safe (Beckman Instruments, Fullerton, CA) and counted in a liquid scintillation counter.

[³H] Nisoxetine Binding to Human Norepinephrine Transporter. Radioligand binding assays are performed by a modification of the method of Jayanthi et al. (1993) in binding buffer containing 50 mM Tris, 300 mM NaCl and 5 mM KCl (pH 7.4). [³H]nisoxetine (specific activity 85.0 Ci/mmol, from Amersham, Arlington Hts., IL) at 0.5 nM is incubated with competing drugs and 25 μ g/tube membrane protein for 60 min at 22°C. Nonspecific binding is determined in the presence of 1 μ M nisoxetine. The remainder of the assay is exactly as described above.

[³H] WIN35428 Binding to Human Dopamine Transporter. Radioligand binding assays are performed using a modification of the method of Pristupa et al. (1994) in a binding buffer containing 50 mM Tris and 120 mM NaCl (pH 7.4). [³H]WIN35428 (Dupont New England Nuclear, Boston MA, specific activity 83.5 Ci/mmol) at 1 nM is incubated with competing drugs and 30 μ g/tube membrane protein for 120 min at 4°C. Nonspecific binding is determined in the presence of 10 μ M WIN35428. The remainder of the assay is exactly as described above.

EVALUATION

The data are analyzed using the LIGAND program (Munson and Rodbard 1980) for calculation of *K_D* values and Hill coefficients.

MODIFICATIONS OF THE METHOD

Using the same methods, Tatsumi et al. (1999) described the pharmacological profile of several neuroleptics at human monoamine transporters.

Gu et al. (1994) constructed stable cell lines expressing transporters for dopamine, norepinephrine and serotonin using parental LLC-PK₁ cells which do not express any of these neurotransmitter transporters.

Meltzer et al. (1997) described inhibitors of monoamine transporters using dopamine- and serotonin-transporter assays. Membranes were prepared from coronal slices from caudate-putamen of brain from adult cynomolgus monkeys.

Owens et al. (1997) measured the affinity of several antidepressants and their metabolites for the rat and human serotonin and norepinephrine transporters.

Inazu et al. (1999) characterized dopamine transport in cultured rat astrocytes.

Siebert et al. (2000) used rat neuronal cultures and transfected COS-7 cells to characterize the interaction of haloperidol metabolites with neurotransmitter transporters.

Sato et al. (2000) studied the selective inhibition of monoamine neurotransmitter transporters by synthetic local anesthetics using cloned transporter cDNAs with transient functional expression in COS cells and stable expression in HeLa cells.

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E.6.1.5

Antagonism of p-Chloramphetamine Toxicity by Inhibitors of Serotonin Uptake

PURPOSE AND RATIONALE

p-Chloramphetamine causes selective toxicity to serotonin neurons. At 10 mg/kg i.p. p-chloramphetamine (p-CA) causes long-term decreases in 5-HT, 5HIAA, [³H]-5-HT-uptake and tryptophan hydroxylase. After acute administration of p-CA, serotonin uptake into synaptosomes isolated from whole brain is reduced for several hours and returns to control values between 1 and 2 days, after which there is a marked long-lasting decrease due to toxic destruction of 5-HT neurons. The initial behavioral effects of p-CA are due to release of dopamine and serotonin. The serotonergic toxicity of p-CA requires active transport into 5-HT neurones where a cytotoxic intermediate compound is formed. Therefore, compounds which block 5-HT uptake will prevent this toxicity. Antagonism of the long-term p-CA induced reduction of synaptosomal ³H-5-HT *in vitro* uptake is a highly useful index of a compound’s ability to inhibit 5-HT re-uptake *in vivo*.

PROCEDURE

Reagents

Two mg/ml dextrose and 0.30 mg/ml iproniazid phosphate are added to Krebs-Henseleit bicarbonate buffer. The mixture is aerated for 1 h with carbogen.

Serotonin creatinine sulfate (Sigma Chemical Co) as a 0.1 mM stock solution in 0.01 N HCl is used to dilute the specific activity of the radiolabeled 5-HT.

A solution of 62.5 nM ³H-5-HT (5-[1,2-³H(N)]-hydroxytryptamine creatinine sulfate, specific activity 20–30 Ci/mmol, New England Nuclear) is prepared in Krebs-Henseleit bicarbonate buffer.

(d,l)-p-Chloramphetamine (Regis Chemical Co) is dissolved in 0.9% NaCl at a concentration of 10 mg/ml.

Drug Treatment

Groups of 8 male Wistar rats, weighing 150–200 g are injected intraperitoneally with saline or molar equivalent doses of the test drug. For initial studies, the dose given is 37.5 µmol/kg (equivalent to 10 mg/kg desipramine base). After 30 min, 4 rats from each group are injected with saline or with p-CA 10 mg/kg i.p. Three days after treatment, the rats are sacrificed. For multiple dosing, the rats are pretreated with the test drug twice up to 4 times in 2 h intervals.

Tissue Preparation

The rats are decapitated and the brains rapidly removed. Whole brain minus cerebellum is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem homogenizer. The homogenate is centrifuged at 1000 g at 0–4°C for 10 min. The crude synaptosomal supernatant is decanted and used for uptake experiments.

Assay

800 µl Krebs-Henseleit bicarbonate buffer+[³H]-5-HT

200 µl Tissue suspension

Tubes are incubated at 37°C under a 95% O₂/5% CO₂ atmosphere for 5 min. For each assay, 3 tubes are incubated at 0°C in an ice bath. After incubation all tubes are immediately centrifuged at 4000 g at 0–4°C for 10 min. The supernatant fluid is aspirated and the pellets dissolved by adding 1 ml of solubilizer (Triton X-100 and 50% ethanol, 1:4, v/v). The tubes are vigorously vortexed, decanted into scintillation vials, and counted in 10 ml of aqueous (Liquiscint) scintillation counting cocktail. Active uptake is the difference between cpm at 37°C and 0°C.

EVALUATION

The percent protection is calculated according to the following formula:

$$\% \text{ protection} = \frac{\text{cpm}[(\text{sal}/\text{sal}) - (\text{sal}/\text{pCA})]}{\text{cpm}[(\text{sal}/\text{sal}) - (\text{sal}/\text{pCA})]} - \frac{\text{cpm}[(\text{sal}/\text{sal}) - (\text{drug}/\text{pCA})]}{\text{cpm}[(\text{sal}/\text{sal}) - (\text{sal}/\text{pCA})]}$$

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E.6.1.6
Receptor Subsensitivity After Treatment with Antidepressants: Simultaneous Determination of the Effect of Chronic Anti-Depressant Treatment on β-Adrenergic and 5-HT₂ Receptor Densities in Rat Cerebral Cortex
PURPOSE AND RATIONALE

The catecholamine and indolamine systems are thought to be involved in affective disorders such as depression. The effect of antidepressants on biogenic amine re-uptake *in vitro* is immediate; whereas the onset of clinical activity is delayed and parallels more closely the time course of receptor changes measured in animal studies. Therefore, the experiment is designed to determine the *in vivo* effects of chronic (10 days) treatment with known and potential antidepressants on the β-receptor, as a measurement of noradrenergic interaction, and on the 5-HT₂ receptor as a measurement of serotonergic interaction in the rat brain. Both receptor densities are measured in cortical tissue from the same animal and compared after treatment with test compounds at doses similar to those of standard drugs.

PROCEDURE**Drug Treatment**

Groups of 4 male Wistar rats, receiving food and water at libitum are maintained on a 12-h diurnal light cycle and given i.p. injections twice daily for 10 days with saline or molar equivalent doses of the experimental drugs (equivalent to 10 mg/kg imipramine). Twenty-four hours after the last dose, the rats are decapitated and the cerebral cortices split along the mid-sagittal sinuses. One half is used for the (³H)-DAH assay and the other half is used for the (³H)-spiroperidol assay. This protocol allows the determination of effects of antidepressants on β-receptors and 5-HT₂-receptors in cerebral cortical tissue from the same animal.

Reagents

- (–)-[propyl-1,2,3-³H]Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
- (±)-propranolol HCl is obtained from Ayerst.
- [Benzene-³H]spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear.

Tissue Preparation

³H-Dihydroalprenolol (³H-DHA) Binding. The cerebral cortices are dissected free, weighed and homogenized in 50 vol of ice-cold 0.05 M Tris buffer, pH 8.0. This homogenate is centrifuged at 40,000 g and the supernatant decanted. The pellet is resuspended and again centrifuged at 40,000 g. The final pellet is resuspended in 0.05 M Tris buffer, pH 8.0. This tissue suspension is then stored on ice until use. The final tissue concentration is 10 mg/ml.

³H-Spiroperidol Binding. The cerebral cortices are dissected, weighed and homogenized in 50 vol of 0.05 M Tris buffer, pH 7.7, and then centrifuged at 40000g for 15 min. The supernatant is discarded, the pellet resuspended and again centrifuged at 40,000 g. The final pellet is resuspended in 50 vol of 0.05 M Tris buffer, pH 7.7 and stored in an ice bath. The final concentration in the assay is 10 mg/ml.

Assay**³H-DAH Binding**

- 380 μl H₂O
- 50 μl 0.5 M Tris buffer pH 8.0
- 20 μl vehicle (for total binding) or 50 μM propranolol (for nonspecific binding)
- 50 μl ³H-DAH stock solution
- 500 μl tissue suspension

The tissue homogenates are incubated for 15 min at 25°C with varying concentrations of ³H-DHA (0.25–4.0 nM). With each ligand concentration triplicate samples are incubated with 1 μM propranolol under identical conditions to determine nonspecific binding. The total added ligand is determined by counting 50 μl of each (³H)-DHA concentration. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice cold 0.05 M Tris buffer, pH 8.0. The filters are counted in 10 ml liquid scintillation cocktail.

³H-Spiroperidol Binding

- 50 μl 0.5 M Tris-physiological salts
- 380 μl H₂O
- 20 μl H₂O (for total binding) or 0.25 mM methysergide (for nonspecific binding)
- 50 μl ³H-spiroperidol stock solution
- 500 μl tissue suspension

The tissue homogenates are incubated for 10 min at 37°C with varying concentrations of ³H-spiroperidol (0.1–3.0 nM). With each ligand concentration, triplicate samples are incubated in the presence of 5 μM methysergide under identical conditions to determine nonspecific binding. The total added ligand is determined by counting 50 μl of each (³H)-spiroperidol concentration. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice cold 0.05 M Tris buffer, pH 7.7. The filters are transferred to scintillation vials and counted in 10 ml of liquid scintillation cocktail.

EVALUATION

Specific binding is defined as the difference in CPM in the presence or absence of excess “cold” ligand. The free ligand concentration is the difference between the total added and the specifically bound fraction at each concentration. The equilibrium binding constants (K_d and B_{max}) are determined by Scatchard analyses using least square regression analysis of the binding data. The Scatchard plot shows “bound/free” versus “bound”. The K_d value is the reciprocal of the slope and B_{max} is the x-intercept. Significant differences of drug treatment are determined by either Dunnett’s or Tukey’s test after one way analysis of variance.

MODIFICATIONS OF THE METHOD

Buckett et al. (1988) found a rapid down-regulation of β -adrenoceptors in brains of rats after 3 days of oral treatment with sibutramine.

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E.6.1.7

Measurement of β -Adrenoreceptor Stimulated Adenylate Cyclase

PURPOSE AND RATIONALE

Noradrenaline stimulates the β -adrenoreceptor linked adenylate cyclase in rat brain. Reduction of this stimulation after treatment with antidepressants is an indicator for receptor down-regulation.

PROCEDURE

Groups of male Sprague-Dawley rats are treated intraperitoneally twice daily for 14 days with saline, 10 mg/kg desimipramine or the test compound. The rats are decapitated and frontal cortices are removed, and placed into ice-cold Krebs physiological buffer (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl $_2$, 2 mM KH $_2$ PO $_4$, 2 mM MgSO $_4$, 25 mM NaHCO $_3$, 100 μ M Na $_2$ S $_2$ O $_5$, 25 μ M EDTA, and 10 mM glucose), pH 7.4. Cortices are cut 350 \times 350 μ at right angles, added to 20 ml Krebs physiological buffer in 50 ml conical flasks, and separated by vortex mixing. The slices are incubated at 37°C for 1 h in a shaking water bath with the buffer replaced by freshly oxygenated medium every 15 min. After this step, 20 μ l [3 H]adenine is added to each flask. The flasks are capped, and the slices are incubated for an additional 30 min at 37°C. This medium is then discarded and the slices are washed with 20 ml fresh buffer. Most of the medium is removed and 30 μ l aliquots of the packed slices are pipetted into tubes containing 300 μ M 3-isobutyl-1-methylxanthine in 270 μ l Krebs physiological buffer. After incubation at 37°C for 15 min, 30 μ l of noradrenaline (final concentration 100 μ M) or 30 μ l buffer are added to each tube and incubated for an additional 10 min. The reaction is stopped by addition of 0.5 ml 1 M HCl. The tubes are placed in ice for 10 min before addition of 0.5 ml NaOH and then centrifuged at 3000 g for 10 min at 4°C. The supernatants are transferred to tubes containing [14 C]-cyclic AMP (approximately 10,000 cpm) to monitor the recovery of [3 H]-cyclic AMP. The radio-labelled cyclic nucleotides are sep-

arated by two-stage column chromatography on alumina neutral WN-3 and Dowex AG 50W-X4 anion exchange resin.

EVALUATION

The activity of adenylate cyclase is calculated as the conversion of [³H]-adenine to [³H]-cyclic AMP. After treatment with antidepressants, this conversion rate is not altered without stimulation by noradrenaline, but significantly reduced in slices treated with the maximally stimulating concentration of 100 μM noradrenaline.

CRITICAL ASSESSMENT OF THE METHOD

Determination of β-adrenoreceptor stimulated adenylate cyclase is another parameter for measurement of down-regulation of adrenoreceptors by chronic treatment with antidepressants.

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E.6.1.8

[³H]Yohimbine Binding to α₂-Adrenoceptors in Rat Cerebral Cortex

PURPOSE AND RATIONALE

This binding assay is used to investigate the interaction of compounds at central α₂ receptors and may indicate possible modes of action for antidepressant, anti-hypertensive and other classes of compounds.

[³H]Yohimbine is a selective antagonist for α₂-receptors (Starke et al. 1975). The use of an antagonist radioligand avoids the complexity of saturation curves that can be observed with radio-labeled agonist ligands. Furthermore, a [³H]antagonist label for the α₂ receptor permits a better evaluation of α₂/α₁ selectivity than a [³H]agonist label, since α₁ affinities are measured with a [³H]antagonist ([³H]WB 4101).

Chronic treatment with desipramine has been shown to decrease the binding of [³H]DHA to rat brain cortical β-receptors. Some investigators have reported that co-administration of yohimbine causes β-receptor down-regulation to occur after fewer antidepressant treatments (Johnson et al. 1980; Scott and Crews 1983). Therefore, it may be of interest to investigate compounds with yohimbine-like properties as antidepressant candidates themselves or in conjunction with antidepressant candidates.

PROCEDURE

Reagents

- 20-fold concentrated buffer:
 - 2.36 M NaCl 137.92 g/liter
 - 100 mM KCl 7.45 g/liter
 - 200 mM glucose 36.03 g/liter in 0.5 M Tris, pH 7.4
- 2-fold concentrated buffer:
 - fold dilution of reagent 1 in deionized water
- Standard buffer:
 - 20-fold dilution of reagent 1 in deionized water
- [Methyl-³H] yohimbine (72–86 Ci/mmol) is obtained from New England Nuclear
 - For IC₅₀ determinations: [³H] Yohimbine is diluted to 40 nM in deionized H₂O
 - Fifty μl of this solution is a 2-ml reaction volume gives a final concentration of 1 nM
- L-NE bitartrate is made up to 10 mM in 0.01 N HCl. 20 μl of this solution gives a final concentration of 100 μM in 2 ml of reaction mixture.

6. Test compounds. For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-4} to 10^{-7} M. Six or seven concentrations are used routinely.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The cortices are weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle (0.009–0.010 cm clearance). The homogenate is centrifuged at 1000 g for 10 min (approximately 3000 rpm using the Sorvall RC-5 centrifuge with heads SS-34 or SM-24). The supernatant is then recentrifuged at 17,000 g for 20 min (approximately 12,500 rpm using the Sorvall RC-5 centrifuge, and heads SS-34 or SM-24). The pellet (P₂) is resuspended in the original volume of 0.32 M sucrose and stored on ice.

Binding Assay

| | |
|--------------|------------------------------------------|
| 430 μ l | H ₂ O |
| 1000 μ l | 2-fold concentration buffer (reagent 2) |
| 20 μ l | drug or 10 mM L-NE bitartrate or vehicle |
| 50 μ l | ³ H-yohimbine (reagent 4) |
| 500 μ l | tissue |

Tubes are vortexed and incubated at 25°C for 10 min. Bound [³H]yohimbine is captured via filtration under reduced pressure. The filters are washed three times with 5 ml aliquots of buffer (reagent 3). The filters are then counted in 10 ml of Liquiscint scintillation fluid.

EVALUATION

Specific binding of [³H]yohimbine is the difference between total bound (in the presence of vehicle) and non-specifically bound (in the presence of 100 μ M L-NE bitartrate). Percent inhibition of specific [³H]yohimbine binding is calculated for each concentration of test drug and *IC*₅₀ values determined by computer-derived log-probit analysis.

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E.6.1.9

Test for Anticholinergic Properties by [³H]-QNB Binding to Muscarinic Cholinergic Receptors in Rat Brain

PURPOSE AND RATIONALE

Several tricyclic antidepressants exert considerable anti-cholinergic effects which limit the therapeutic use in some patients. Amitriptyline has the greatest incidence of these side effects and is the most potent in binding to muscarinic receptors. Desipramine, which exhibits less incidence of atropine like side effects shows a lower affinity for muscarinic receptors. Since there is no evidence that the anticholinergic effects contribute to the therapeutic efficacy, antidepressant drugs with low anti-cholinergic effects are desired.

Quinuclidinyl benzilate (QNB) is a specific muscarinic cholinergic antagonist in both peripheral and central tissues. The binding characteristics of ³H-QNB were first described by Yamamura and Snyder (1974), who showed that this ligand was displaced by muscarinic antagonists, but not by nicotinic or non-cholinergic drugs. The levorotatory isomer being more potent than the racemate is used as the radioactive ligand.

PROCEDURE

Reagents

L-[Benzilic-4,4'-³H]-quinuclidinyl benzilate (30–40 Ci/mmol) is obtained from New England Nuclear. For *IC*₅₀ determinations [³H]-QNB is made up to a concentration of 40 nM and 50 μ l is added to each tube (yielding a final concentration of 1 nM in the assay).

Atropine sulfate is obtained from Sigma Chemical Co.

Test compounds: For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

Male Wistar rats are killed by decapitation and their brains rapidly removed. After removal of the cerebellum, each brain is homogenized in 10 volumes of ice-cold 0.32 M sucrose in a Potter-Elvehjem glass homogenizer. The homogenate is then centrifuged at

1000 g for 10 min and the pellet discarded. The resultant supernatant is further dispersed and used for [³H]-QNB binding studies. Specific binding is 20% of the total added ligand and approximately 95% of the total bound ligand.

Assay

| | |
|---------|------------------------------------------------------------------------------------------------------------------------------------------|
| 100 µl | 0.5 M Na/K phosphate buffer, pH 7.4 (134 g Na ₂ HPO ₄ · 7H ₂ O + 68 g KH ₂ PO ₄) |
| 780 µl | H ₂ O |
| 50 µl | ³ H-QNB stock solution |
| 20 µl | vehicle (for total binding) or 2 mM atropine (for nonspecific binding) |
| 1000 µl | 0.05 M Na/K phosphate buffer (1:10 diluted) pH 7.4 |
| 50 µl | tissue suspension |

Tissue homogenates are incubated for 60 min at 25°C with 1 nM ³H-QNB and varying drug concentrations. With each binding assay, triplicate samples containing 2 µM atropine sulfate are incubated under identical conditions to determine nonspecific [³H]-QNB binding. After incubation, the samples are cooled and then rapidly filtered through glass filters (Whatman GF/B) under reduced pressure. The filters are washed 3 times with 5 ml of ice-cold phosphate buffer and then placed in scintillation vials. After the addition of 10 ml of counting cocktail, radioactivity is assayed by liquid scintillation spectrophotometry.

EVALUATION

Specific [³H]-QNB binding is determined by the difference between total [³H]-QNB and bound radioactivity in the presence of 2 µM atropine sulfate. Data are converted to percent specific bound [³H]-QNB displaced by test drugs and *IC*₅₀ values obtained from computer derived log-probit analysis. The inhibition at each drug concentration is the mean of triplicate determinations.

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E.6.1.10

Monoamine Oxidase Inhibition: Inhibition of Type A and Type B Monoamine Oxidase Activities in Rat Brain Synaptosomes

PURPOSE AND RATIONALE

The mood-elevating effects of the antituberculosis drug iproniazid have been observed clinically. The mode of action was elucidated to be the inhibition of the enzyme monoamine oxidase. This was followed by wide use of monoamine oxidase inhibitors for the treatment of depression. However, side effects due to interaction with dietary amines have been observed. The biological role of monoamine oxidase is to regulate the levels of endogenous amines (norepinephrine, dopamine and serotonin) and exogenously administered amines. Based on different substrate and inhibitor specificities two forms of monoamine oxidase (A and B) were described. Dopamine and tyramine are substrates for both types, serotonin and epinephrine are substrates for type A, and β-phenylethylamine and benzylamine are substrates for type B. Iproniazid and tranylcypromine are nonselective inhibitors, clorgyline is a selective inhibitor of type A, deprenyl and pargyline are selective inhibitors of type B. It has been suggested that treatment with selective blockers of type B results in less detrimental food interactions.

PROCEDURE

Tissue Preparation

Male Wistar rats weighing 150–250 g are sacrificed and the brains rapidly removed. Whole brain minus cerebellum is homogenized in 9 volumes of ice-cold, phosphate-buffered 0.25 M sucrose, using a Potter-Elvehjem homogenizer. The homogenate is centrifuged at 1000 g for 10 min and the supernatant decanted and recentrifuged at 18,000 g for 20 min. The resulting pellet (P₂) is resuspended in fresh 0.25 M sucrose and recentrifuged at 18,000 g for 20 min. The washed pellet is resuspended in the original volume

of 0.25 M sucrose and serves as the tissue source for mitochondrial monoamine oxidase.

Assay

- 50 μ l 0.5 M PO₄ buffer, pH 7.4
 450 μ l H₂O
 100 μ l H₂O or appropriate drug concentration
 200 μ l tissue suspension

The tubes are preincubated for 15 min at 37°C and the assay is started by adding 100 μ l of substrate (¹⁴C-5-HT or ¹⁴C β -phenylethylamine) at 10 s intervals. The tubes are incubated for 30 min at 37°C and the reaction is stopped by the addition of 0.3 ml of 2 N HCl. Tissue blank values are determined by adding the acid before the substrate. Seven ml of diethylether are added, the tubes are capped and shaken vigorously for 10 min to extract the deaminated metabolites into the organic phase, which is separated from the aqueous phase by centrifugation at 1000 g for 5 min. A 4 ml aliquot of the ether layer is counted in 10 ml of liquid scintillation counting cocktail.

EVALUATION

The percent inhibition at each drug concentration is the mean of triplicate determinations. IC₅₀ values are determined by log-probit analyses.

For example, deprenyl shows IC₅₀-values of 3.9×10^{-6} against MAO A and 3.0×10^{-8} against MAO B.

MODIFICATION OF THE METHOD

Colzi et al. (1992) measured dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) outflow after a reversible MAO-A inhibitor with a brain microdialysis technique in rats.

Frankhauser et al. (1994) tested the interaction of MAO inhibitors and dietary tyramine by measurement of peak systolic blood pressure in conscious rats. The increase of blood pressure after oral application of tyramine was potentiated by pretreatment with MAO inhibitors. Reversible MAO inhibitors could be differentiated from non-selective, irreversible MAO inhibitors.

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E.6.2

In Vivo Tests

E.6.2.1

Catalepsy Antagonism

PURPOSE AND RATIONALE

Observations about **cataleptic behavior in chicken** have first been described more than 300 years ago (Schwenter 1636; Kircher 1646), and again reported about 100 years ago (Czermak 1873; Heubel 1877; Verworn 1898). This phenomenon was used by Vogel and Ther (1963) as a simple method to detect antidepressants besides other central stimulants.

PROCEDURE

Adult white Leghorn chicken are used. The animal is grasped with both hands whereby the left hand pushes the chicken slightly down and the right hand supports the animal from the ventral side. Immediately, the chicken is turned on its back and hold with the

right hand for 1 min. Usually, cataleptic numbness occurs immediately. The cataleptic state can be sustained by slight pushing the head of the animal on the table. After 1 min the right hand is carefully withdrawn. The chicken remains in the cataleptic state for several min up to 1 h. The cataleptic rigor is interrupted by noise or fast movements of the observer. Clapping of the hands above the head arouses the chicken which jumps up and runs away. The chicken is always aroused by pulling on the wings. The animals are pretested in order to be sure about the cataleptic behavior of an individual chicken. As already found by previous investigators, the experiment can be repeated several times. Control studies showed that in untreated animals the phenomenon could be elicited 6 times every 30 min during a period 5 days. After the control experiments, the animals are injected i.p. with the test compound or the vehicle. The test is performed 4 times every 30 min during 2 h.

EVALUATION

The test is considered to be positive if the cataleptic rigor does not occur after treatment or is interrupted spontaneously within 1 min at least twice during the 2 h test period. The suppression of the cataleptic phenomenon is the criterion for a positive response. Furthermore, the arousal after hand-clapping or pulling on the wings is recorded in order to register central sedative effects. In order to obtain dose-response curves 12 animals per group are treated with various doses. ED_{50} values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The specificity of the method has been tested. Antidepressant agents like imipramine and ethyltryptamine-acetate or other monoamino-oxidase-inhibitors and d-desoxyephedrine show a dose dependent effect. Moreover, the effects were dependent on time. ED_{50} values decreased on consecutive days after imipramine and monoamino-oxidase inhibitors but not after d-desoxyephedrine. Therefore, the test can be considered as specific for central stimulants allowing the possibility to distinguish between antidepressants and central stimulants of the amphetamine type.

MODIFICATIONS OF THE METHOD

Simiand et al. (2003) and Gabriel et al. (2005) described **tonic immobility in gerbils** as a model for detecting antidepressant-like effects. When grasped by the skin of the nape and lifted into the air, the young of many altricial mammals exhibit an immobility response in which they tuck their limbs against their

bodies and remain inert. Such a response can be induced also in adults of some species, such as Mongolian gerbils (De la Cruz and Junquera 1993). To test antidepressant-like effects in the tonic immobility paradigm in gerbils, animals (six to nine per group) were held on a flat surface and were firmly pinched for 15 s at the scruff of the neck using the thumb and the index finger. They were then placed on parallel bars (4 mm in diameter, 28 cm long, spaced 5 cm apart and having a 3-cm difference in height). The front paws were gently placed on the upper bar and the hind paws on the lower bar. The duration of tonic immobility was measured in five successive trials with a 30-s inter-trial interval. Each trial ended when an animal started to move or after 90 s of immobility. Experiments were started 30 min after i.p. administration of test drug.

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E.6.2.2

Despair Swim Test

PURPOSE AND RATIONALE

Behavioral despair was proposed as a model to test for antidepressant activity by Porsolt et al. (1977, 1978). It was suggested that mice or rats forced to swim in a re-

stricted space from which they cannot escape are induced to a characteristic behavior of immobility. This behavior reflects a state of despair which can be reduced by several agents which are therapeutically effective in human depression.

PROCEDURE

Male Sprague-Dawley rats weighing 160–180 g are used. They are brought to the laboratory at least one day before the experiment and are housed separately in Makrolon cages with free access to food and water. Naive rats are individually forced to swim inside a vertical Plexiglas cylinder (height: 40 cm; diameter: 18 cm, containing 15 cm of water maintained at 25°C). Rats placed in the cylinders for the first time are initially highly active, vigorously swimming in circles, trying to climb the wall or diving to the bottom. After 2–3 min activity begins to subside and to be interspersed with phases of immobility or floating of increasing length. After 5–6 min immobility reaches a plateau where the rats remain immobile for approximately 80% of the time. After 15 min in the water the rats are removed and allowed to dry in a heated enclosure (32°C) before being returned to their home cages. They are again placed in the cylinder 24 h later and the total duration of immobility is measured during a 5 min test. Floating behavior during this 5 min period has been found to be reproducible in different groups of rats. An animal is judged to be immobile whenever it remains floating passively in the water in a slightly hunched but upright position, its nose just above the surface. Test drugs or standard are administered one hour prior to testing. Since experiments with the standard drug (imipramine) showed that injections 1, 5 and 24 h prior the test gave the most stable results in reducing floating these times are chosen for the experiment.

EVALUATION

Duration of immobility is measured in controls and animals treated with various doses of a test drug or standard. Antidepressant drugs, but also stimulants like amphetamine and caffeine, reduce duration of immobility. Dose-responses can be evaluated.

CRITICAL ASSESSMENT OF THE METHOD

The method, also called “forced swim test,” has been used and modified by many authors, e. g. Kauppila et al. (1991, van der Heyden et al. 1991).

Advantages of the method are the relative simplicity and the fact that no interaction with other drugs is necessary. Like in other behavioral tests, e. g. the catalepsy test in chicken, not only antidepressants and

monoamino-oxidase inhibitors but also central stimulants give positive results.

In a critical review of the forced swimming test, Borsini and Meli (1988) discussed the various modifications and proposed rats to be more suitable than mice for detecting antidepressant activity.

The model is purely behavioral without presuppositions concerning the mechanism of action of potential antidepressants. It is sensitive to a large number of atypical antidepressants otherwise inactive in the more classical tests. The rat version seems to be more selective (fewer false positives) and the mouse version more sensitive (fewer false negatives) (Porsolt et al. 1991).

Natoh et al. (1992) reviewed the theoretical background of the forced swimming test and the various factors which can possibly affect the results.

MODIFICATIONS OF THE METHOD

Wallach and Hedley (1979) reported positive results with antihistamines and Giardina and Ebert (1989) with an ACE-inhibitor. Differentiation is achieved by the simultaneous evaluation of motor activity.

Cervo and Samanin (1987) suggested potential antidepressant activities of a selective serotonin_{1A} agonist based on anti-immobility activity in the forced swimming test in rats without effect on open-field activity.

Nishimura (1988, 1989, 1993) published a modification of the forced swim test using straw suspension in the water tank. The apparatus used was a vertical glass cylinder (height: 40 cm, diameter: 18 cm) equipped with 4 pieces of straw (length 24 cm, diameter: 0.4 cm) that were suspended from above. The cores of the straws were filled with cotton rope. The straws were painted black from the surface of the water to a height of 10 cm. The apparatus was filled with water to a height of 15 cm and maintained at 25°C. Thirty min after treatment with drugs or saline, the rats were placed in the apparatus without straw suspension and the total duration of immobility measured for a period of 5 min. Immediately thereafter, 4 pieces of straw were suspended and the total duration of immobility in the following 5-min observation period again measured.

In an effort to enhance the sensitivity of the traditional forced swim test in the rat so that it can be responsive to specific serotonin reuptake inhibitors, Lucki (1997) proposed several simple procedural modifications. These developments include increasing the water depth to 30 cm from the traditional 15–18 cm, and using a time sampling technique to rate the predominant behavior over a 5-s interval. Spe-

cific components of active behavior can be distinguished: (1) climbing behavior (also known as trashing), which is defined as upward-directed movements of the forepaws along the side of the swim chamber; (2) swimming behavior, the movement (usually horizontal) throughout the swim chamber that also includes crossing into another quadrant; and (3) immobility, which is defined as when no additional activity is observed other than that required to keep the rat's head above the water. As a result of the increase in water depth, there is considerably less immobility than in the traditional test because the animals cannot have contact with the cylinder bottom. The major advantage of this modified test is that it reveals that catecholaminergic agents decrease immobility with a corresponding increase in climbing behavior, whereas 5-HT-related compounds, such as specific serotonin reuptake inhibitors, also decrease immobility but increase swimming behavior.

López-Rubalcava and Lucki (2000) described strain differences in the behavioral effects of antidepressant drugs in the rat forced swim test when the noradrenergic antidepressant, desipramine, and the serotonergic antidepressants fluoxetine and 8-OH-DPAT were tested in Wistar–Kyoto rats and Sprague Dawley rats.

Sun and Alkon (2003, 2004) described the **open space swimming test** to index antidepressant activity. Rats were placed individually in a round pool, with a diameter of 152 cm and a height of 60 cm and was filled with 40 cm water at 21°C. Rats were free to swim (or not to swim) for 15 min and then returned to their home cage. The same procedure was followed 24 h later for 3 days. The swimming/drifted path was recorded with a video tracking system. The measurement is considered to be more objective than the forced swimming test. The procedure induces depressive behavior and impairs learning and memory in the rats.

Galea et al. (2001) found that estradiol alleviates depressive-like symptoms in an animal model of post-partum depression. The effect of hormone withdrawal following hormone-simulated pregnancy on depressive-like behavior in the forced swim test was investigated in female Long-Evans rats.

Using the forced swim test or elevated plus-maze, Stoffel and Craft (2004) studied ovarian hormone withdrawal-induced “depression” in female rats.

Gregus et al. (2005) used the forced swim test to study the effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats.

Buckett et al. (1982) described an automated apparatus for behavioral testing of typical and atypical an-

tidepressants in **mice**. A multichannel system can test 10 mice simultaneously. Each mouse is placed in the beam of a Doppler radar head and horn assembly. The moving mouse causes reflections of a frequency differing from the transmitted signal. Within the Doppler head these reflected waves are mixed with a proportion of transmitted waves to produce a difference signal proportional to the activity of the mouse within the beam. The output of each Doppler head is fed to an amplifier whose gain has been calibrated to compensate for differences in sensitivity between individual heads. The method is claimed to eliminate human error and bias and to allow the testing of large numbers of compounds.

Alpermann et al. (1992) used a slightly modified behavioral despair test in mice. Sixty min after administration of the test compounds, the animals are placed in glass cylinders containing water up to a height of 10 cm (water temperature 22–24°C). From the second minute onward, immobility of each mouse is rated every 30 s. After 10 observations mean values and standard deviations in each treatment group are calculated. Compared with the immobility score of the control group percent reduction can be calculated.

Nomura et al. (1982) published a modification of the despair swim test in mice involving a small water wheel set in a water tank. Mice placed on this apparatus turned the wheel vigorously but, when they abandoned attempts to escape from the water, the wheel stopped turning. The number of rotations of the water wheel were counted.

Hata et al. (1995) studied the behavioral characteristics of SART (specific alterations of rhythm in temperature)-stressed mice in forced swimming tests and evaluated the effects of anxiolytics and antidepressants.

Weiss et al. (1998) reported selective breeding of rats for high and low motor activity in the swim test. Sprague Dawley rats were selectively bred for low motor activity (low struggling time/high floating time), while others were bred for high motor activity (high struggling time/low floating time).

Lucki et al. (2001) investigated the sensitivity to desipramine, a selective norepinephrine reuptake inhibitor, and fluoxetine, a selective serotonin reuptake inhibitor, in seven inbred and four outbred mouse strains. Considerable differences in the results of the forced swim test were found.

Sachdev et al. (2002) used the forced swim model of depression in rats to investigate the antidepressant effect of different frequencies of transcranial magnetic stimulation.

Krahe et al. (2002) tested the effects of rotational side preferences in immobile behavior of normal mice in the forced swimming test.

Buckley et al. (2004) used both the mouse forced swim test and the rat forced swim test to characterize a nicotinic agonist.

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E.6.2.3

Tail Suspension Test in Mice

PURPOSE AND RATIONALE

The “tail suspension test” has been described by Steru et al. (1985) as a facile means of evaluating potential antidepressants. The immobility displayed by rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in turn may reflect depressive disorders in humans. Clinically effective antidepressants reduce the immobility that mice display after active and unsuccessful attempts to escape when suspended by the tail.

PROCEDURE

Male Balb/cJ mice weighing 20–25 g are used preferentially. They are housed in plastic cages for at least 10 days prior to testing in a 12 h light cycle with food and water freely available. Animals are transported from the housing room to the testing area in their own cages and allowed to adapt to the new environment for 1 h before testing. Groups of 10 animals are treated with the test compounds or the vehicle by intraperitoneal injection 30 min prior to testing. For the test the mice are suspended on the edge of a shelf 58 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility is recorded for a period of 5 min. Mice are considered immobile when they hang passively and completely motionless for at least 1 min.

EVALUATION

The percentage of animals showing the passive behavior is counted and compared with vehicle treated controls. Using various doses, ED_{50} values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The tail suspension test has been found to be an easy method to test potential antidepressant compounds. However, it has been reported that several mouse strains are essentially resistant to tail-suspension induced immobility.

MODIFICATIONS OF THE METHOD

Chermat et al. (1986) adapted the tail suspension test to the rat.

Porsolt et al. (1987), Stéru et al. (1987) recommended the use of the automated tail suspension test for the primary screening of psychotropic agents. A specially developed computerized device automatically measures the duration of immobility of 6 mice at one time and at the same time provides a measure of the energy expended by each animal, the power of the movements.

Vaugeois et al. (1996) described a genetic mouse model of helplessness sensitive to imipramine. Lines of mice were selectively bred to diverge in their spontaneous helplessness in the tail-suspension test. By the second generation of selection, only mice of the helpless line were sensitive to the antidepressant imipramine.

Mayorga and Lucki (2001) demonstrated limitations on the use of the C57BL/6 mouse in the tail-suspension test. Under baseline conditions, the majority of C57BL/6 mice climbed up their tails during the 6-

min test session. The occurrence of this behavior was not found in other strains of mice.

Liu and Gershenfeld (2001) found genetic differences in the tail-suspension test among 11 inbred strains of mice as far as basal immobility was concerned. Surprisingly, the reduction of immobility by imipramine was independent of basal immobility.

Ripoli et al. (2003) tested antidepressant-like effects in various mice strains in the tail-suspension test and found considerable differences depending on strain and test drug.

Shearman et al. (2003) tested antidepressant-like effects of a cannabinoid CB1 receptor inverse agonist in mice using an automated tail-suspension apparatus (TSE Systems, Bad Homburg, Germany) with a tail hanger connected to a precision load cell. Mice remained suspended by the tail, at a height of 35 cm from the tabletop, for 6 min. During this time the load cell recorded the mouse's movements and transmitted the information to a computer, which then recorded the rate of immobility within the course of the session, and calculated total duration of immobility.

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E.6.2.4

Learned Helplessness in Rats

PURPOSE AND RATIONALE

Animals exposed to inescapable and unavoidable electric shocks in one situation later fail to escape shock in a different situation when escape is possible (Overmier and Seligman 1967; Maier and Seligman 1976). This phenomenon was evaluated as a potential animal model of depression (Sherman et al. 1979; Martin et al. (1986), Christensen and Geoffroy 1991; Tejedor del Real et al. 1991; Weiss and Kilts 1998).

PROCEDURE

Learned helplessness is produced in male Sprague-Dawley rats (300 g) by exposure to electric shock (0.7 mA) for 1 h on a schedule of 10 s of shock/min. The apparatus is a 30×45×30 cm box with a grid floor. At a height of 20 cm above the floor, a platform (7.5×7.5 cm) can be inserted through one side wall to allow a jump-up escape response. The platform is not available during training. After the appropriate treatment, the animals are tested for acquisition of a jump-up escape in the same apparatus. At the beginning of a trial, the platform is pushed into the box and a 0.4 mA shock initiated. Shock is terminated in 10 s if the animal has not escaped onto the platform by this time. If an escape response occurred, the animal is allowed to remain on the platform for the duration of 10 s, then returned to the grid floor. Ten such trials with an intertrial interval of 20 s are given. In a naive control group of rats, this training resulted in 80% acquiring learned helplessness behavior. Drugs are given before the training and the test period.

EVALUATION

A drug is considered to be effective, if the learned helplessness is reduced and the number of failures to escape is decreased. Imipramine was found to be active only after repeated applications. A benzodiazepine was effective, whereas chlorpromazine was ineffective.

CRITICAL ASSESSMENT OF THE METHOD

The “learned helplessness” in the rat can be regarded as an additional measure for antidepressant activity in addition to other tests. The test is time consuming and

its specificity is questionable. The major drawback of the model is that most of the depression-like symptomatology does not persist beyond 2–3 days following cessation of the uncontrollable shock.

MODIFICATIONS OF THE METHOD

Vaccheri et al. (1984) used an apparatus with an lever to be pressed to interrupt the shock. Giral et al. (1988), Porsolt et al. (1990) Simiand et al. (1992) used shuttle-boxes for escape.

Curzon et al. (1992) described a similar rat model of depression. Rats were tested in an open field after being restrained by taping them to wire grids for two h on the preceding day. The reduced activity is antagonized by antidepressant drugs.

A modification of the test incorporating aspects of chronic mild stress paradigm was described by Gamberana et al. (2001) A model of anhedonia is based on the finding that exposure to repeated unavoidable stress prevents the development of an appetitive behavior induced and maintained by a highly palatable food (vanilla sugar) in rats fed ad libitum (Ghiglieri et al. 1997).

The chronic stress procedure involves restraint and novel housing, and avoids the problems associated with food deprivation (Reid et al. 1997).

Vollmayr et al. (2001) studied brain-derived neurotrophic factor (BDNF) stress responses in rats bred for learned helplessness in a system provided by TSE Systems, Bad Homburg, Germany. Two strains of rats were bred, one which reacts with congenital helplessness to stress, and one which congenitally does not acquire helplessness when stressed. In rats with congenital helplessness, acute immobilization stress does not induce a reduction of BDNF expression in the hippocampus, which is observed in Sprague Dawley rats that do not acquire helplessness.

Vollmayr and Henn (2001) proposed key factors that can be manipulated to enhance both the usability and the reliability of the rat helplessness paradigm.

Rats bred for congenital learned helplessness (King et al. 1993; Shumake et al. 2000) responded less to sucrose but did not show deficits in activity or learning (Vollmayr et al. 2004).

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E.6.2.5

Muricide Behavior in Rats

PURPOSE AND RATIONALE

Horovitz et al. (1965) described a selective inhibition of mouse-killing behavior in rats (Karli 1956; Karli et al. 1969) by antidepressants. The test can be used to evaluate antidepressants such as tricyclics and MAO-inhibitors.

PROCEDURE

Male Sprague-Dawley rats (300–350 g) are isolated for 6 weeks in individual cages. They have access to food and water ad libitum. One mouse is placed into the rat’s cage. About 10 to 30% of rats kill the mouse by biting the animal through the cervical cord. Only rats consistently killing mice within 5 min after presentation are used for the test. The mice are removed 15 to 45 s after they have been killed in order to prevent the rats from eating them. Drugs are injected i.p. to the rats before the test. Mice are presented 30, 60 and 120 min after drug administration.

EVALUATION

Failure to kill a mouse within 5 min is considered inhibition of muricidal behavior. Performing dose-response experiments, the ED_{50} is defined as the dose which inhibits mouse killing in 50% of the rats.

MODIFICATIONS OF THE METHOD

Injections of 5,7-dihydroxytryptamine into the lateral hypothalamus increased mouse-killing behavior in rats (Vergnes and Kempf 1982).

Molina et al. (1985) considered rats isolated for at least one month which do not kill mice in a 30 min test period as spontaneous non-killer rats. In these animals, mouse-killing behavior could be induced by i.p. injection of 150 mg/kg p-chlorophenylalanine daily for 2 days or by electrolytic lesions in the dorsal and median raphe.

McMillen et al. (1988) studied the effects of housing and muricidal behavior on serotonergic receptors and interactions with novel anxiolytic drugs.

CRITICAL ASSESSMENT OF THE METHOD

The mouse-killing behavior is inhibited not only by anti-depressants but also by central stimulants like d-amphetamine (Horovitz et al. 1966; Sofia 1969a, b), some antihistamines (Barnett et al. 1969), and some

cholinergic drugs (McCarthy 1966; Vogel and Leaf 1972; Wnek and Leaf 1972). Neuroleptics and benzodiazepines are active only in doses which impair motor performance.

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duces changes in adult rats that resembles endogenous depression in man, based on earlier observations by Mirmiran et al. (1981), Rodriguez-Echandia and Broitman (1983). This phenomenon has been studied by several research groups in many respects (Neill et al. 1990; Vogel et al. 1990b–e, 1996a, b; Velazquez-Moctezuma and Diaz-Ruiz 1992; Yavari et al. 1993; Maudhuit et al. 1995, 1996; Prathiba et al. 1995, 1997, 1998, 1999; Feenstra et al. 1996; Kinney et al. 1997; Bonilla-Jaime et al. 1998; Dwyer and Rosenwasser 1998).

PROCEDURE

Three days after birth, all male pups of Sprague Dawley mother rats are cross-fostered and all female pups are killed. Cross-fostering consists of removing all pups from their biological mothers and placing them with another lactating female (the non-biological foster mother). Each litter is divided into two groups of approximately equal number. Each half of the litter is placed with a different lactating female (the foster mother), and each foster mother receives half the pups from two litters. All pups with each foster mother receive the same treatment (clomipramine or saline) and the two pup groups of each original litter are assigned to different treatments. Thus, each original pup litter contributes equally to experimental and control groups. Each pup is injected subcutaneously between the shoulder blades with 15 mg/kg clomipramine hydrochloride or saline vehicle twice daily on postnatal days 8 through 21. At one month of age the pups are weaned and housed as litters until approximately 3 weeks prior testing, at which time they are individually housed.

All testing is conducted in a Coulbourn operant conditioning chamber (Lehigh Valley, PA) placed in a quiet area. The floor rods are connected with a shock generator and a shock scrambler.

Behavioral testing is commenced at 3 months postnatally. Each test pair consists of one clomipramine-treated and one vehicle-treated rat. Prior to testing, all clomipramine- and saline-treated rats are paired by body weight (within 5 grams) to diminish size differences that could affect behavioral results. Pairs remain the same throughout the testing.

For evaluation of drugs, clomipramine-treated rats are administered subcutaneously for 4 days twice daily saline, or standard drug (10 mg/kg imipramine), or test drug.

Tests are done daily for 4 days. On the first day, the animals are placed in the chamber for 12 min habituation. On days 2–4, the sessions start with a 2 min

E.6.2.6

Behavioral Changes After Neonatal Clomipramine Treatment

PURPOSE AND RATIONALE

Vogel et al. (1988, 1990a), Hartley et al. (1990) reported that neonatally administered clomipramine pro-

habituation period followed by 10 min of shock delivery. Shocks (1.33 mA, 0.5 s duration) are delivered on a variable schedule with a minimum of 5 and a maximum of 10 s shock intervals. This results in a total of 70–80 shocks within the 10-min session.

Two observers score simultaneously. Each watches one rat of the pair. The rats are identified by a red mark on the fur. The behaviors produced are almost totally in response to shock delivery; both animals are almost immobile between the shocks. Both observers are blind to the treatment conditions of the animals.

Offensive and defensive behaviors are scored including the following 4 behaviors:

1. An upright position which is part of a mutual upright posture in which the dominant rat towers over the submissive rat. The submissive rat rears on its hindlegs, with the head positioned at an upward angle, the forepaws extended toward the attacking animal, and the ventral surface of the body continually facing the opponent.
2. In the offensive crouch, the dominant animal turns its flank towards the subordinate; the submissive crouch is characterized by freezing in a motionless crouching position.
3. Mounting behavior is frequently seen and is the same as seen in the male prior to copulating with a female.
4. Leaps in response to the shock which are directed to the other rat are scored as aggressive responses.

Three defensive or submissive behaviors were observed:

1. Defensive upright posture;
 2. submissive crouch;
 3. a supine position in submission to the dominant rat.
- In this system each offensive behavior by one rat is partly defined by a defensive behavior of the other rat and vice versa.

EVALUATION

The individual behaviors of the treatment and control groups are listed for offensive and defensive behavior on days 1, 2, and 3 and the total of scores calculated for each group. Analysis of variance is applied to offensive, defensive, difference (offensive minus defensive) and total (offensive plus defensive behavior scores). Rats treated postnatally with clomipramine have significantly fewer offensive and significantly more defensive behaviors. This effect is ameliorated by treatment with antidepressant drugs.

CRITICAL ASSESSMENT OF THE TEST

Not only clomipramine, but also other psychotropic drugs induce changes in behavior of adult rats after treatment in neonatal age (Drago et al. 1985; Frank and Heller 1997; Hansen et al. 1998), however, clomipramine induces the most pronounced effects (Velazquez-Moctezuma et al. 1993; Vogel and Hagler 1996; Hansen et al. 1997). Specificity of the procedure to evaluate potential antidepressant compounds remains to be established.

MODIFICATIONS OF THE METHOD

Neonatal treatment with clomipramine not only reduces shock induced aggression, but also induces REM sleep abnormalities (Mirmiran et al. 1981; Vogel et al. 1990b,c; Frank and Heller 1997), decreases intracranial self-stimulation (Vogel et al. 1990d), increases activity in open-field tests in adulthood (Hartley et al. (1990, Prathiba et al. 1997), increases immobility in the forced swim test (Velazquez-Moctezuma and Diaz-Ruiz 1992), reduces adult male rat sexual behavior (Neill et al. 1990; Velazquez-Moctezuma et al. 1993; Vogel et al. 1996; Bonilla-Jaime et al. 1998.), influences the hypothalamic-pituitary-adrenal axis in adult rats (Feenstra et al. 1996; Prathiba et al. 1998, 1999). Vázquez-Palacios et al. (2005) studied antidepressant effects of nicotine and fluoxetine in the animal model of depression induced by neonatal treatment with clomipramine.

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E.6.2.7

Chronic Stress Model of Depression

PURPOSE AND RATIONALE

The chronic mild stress paradigm was developed in order to simulate in animals the symptom of anhedonia, a major feature of depression (Willner et al. 1987, 1992; Forbes et al. 1996; Willner 1997; Willner and Mitchell 2002; Grippo et al. 2003; Konkle et al. 2003; Pijlman et al. 2003; Strekalova et al. 2004). The model has been used to test compounds with antidepressant activity (Moreau et al. 1996; Kopp et al. 1999; Papp and Wieronska 2000; Harkin et al. 2002; Ducottet et al. 2003; Gabriel et al. 2005).

The stress regime was modified by several authors.

The stress regimen used by Moreau et al. (1996) consisted of exposing male Wistar rats each week to a variety of unpredictable, mild stressors such as repeated 1-h periods of confinement to small cages with bells ringing every 10 min, one period of overnight illumination, one overnight period of food and water deprivation immediately followed by 2 h of access to restricted food, one overnight period of water deprivation followed by 1 h exposure to an empty bottle, and one overnight period of group housing in a damp cage.

Grippo et al. (2003) exposed male Sprague Dawley rats to the following stressors in random order: continuous overnight illumination, 40° cage tilt along the vertical axis, paired housing, soiled cage, exposure to an empty water bottle immediately following a period of acute water deprivation, stroboscopic illumination, and white noise.

Ducottet et al. (2003) studied the effects of a selective non-peptide corticotropin-releasing factor receptor 1 antagonist in a mild stress model of depression in **mice**. The method is based on a regimen published by Kopp et al. (1999) and by Griebel et al. (2002a, 2002b).

PROCEDURE

Male BALB/cByJlco mice, aged 9 weeks at the beginning of chronic mild stress experiments, were housed for 1 week individually in small cages with food and water ad libitum at a 12/12 h light/dark cycle and 21°C. The chronic mild stress procedure consists of restraint in a small tube for 1 h, forced bath in water at 32°C for 30 min, water and food deprivation for 15 h, paired housing in damped sawdust for 18 h, food restriction for 3 h, access to an empty water bottle for 2 h, and inversion of the light/dark cycle. Over 3 weeks, four to six of these procedures were applied every day in random order. Physical state was controlled weekly using a scale from 1 to 3 using piloerection, dirty fur, and body weight as symptoms. At the end of the experiment, the mice were subjected to a light/dark test.

Treatment started at week 5, while the stress regimen was continuing. Mice were divided into three groups ($n=20$) each subjected to a 5-week period of chronic mild stress before being administered during a 4-week period with one of the following treatments: vehicle, daily i.p. injections of 10 mg/kg fluoxetine as standard, or test drug.

EVALUATION

Data were analyzed using non-parametric exact analyses including the Monte Carlo correction. Data of physical state were analyzed with a Friedman test, followed by a Wilcoxon signed ranks test comparing initial week with chronic mild stress weeks and treatment weeks. Data of body weight were treated with a Kruskal-Wallis test for each week followed by a permutation test for two independent samples. The light/dark test was submitted to a Kruskal-Wallis test followed by an a posteriori permutation test.

MODIFICATIONS OF THE METHOD

Since the incidence of clinical depression is significantly higher in woman than in men, Konkle et al. (2003) compared the effects of 6 weeks of chronic mild stress administration among female and male rats of Sprague Dawley and of Long Evans strains.

Matthews and Robbins (2003) reported that a specific periodic neonatal maternal separation procedure leads to a robust constellation of behavioral changes in the adult rat that resembles core aspects of anhedonia in men.

Sammot et al. (2001, 2002) studied antidepressant reversal of interferon- α -induced anhedonia utilizing a three-bottle test (Muscat et al. 1991). A drinking and feeding monitoring system (TSE, Systems Bad Homburg, Germany) was used. In this test, three concentra-

tions of sucrose (1%, 8%, and 32%) are simultaneously presented to each individually caged rat. The three-bottle test allows demonstration of the monotonic relationship between intake and hedonic value as is characteristic of human studies.

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E.6.2.8

Novelty-Induced Hypophagia Test

PURPOSE AND RATIONALE

The onset of the therapeutic response to antidepressant treatment exhibits a characteristic delay. While acute antidepressant treatment increases synaptic monoamines within minutes to hours, weeks of sustained treatment are required to induce therapeutic effects (Blier 2003). Few models have been developed in which chronic, but not acute, antidepressant treatment alters behavior (Brodhoff et al. 1988, 1989; Borsini et al. 2002; Cyran et al. 2002; Cyran and Mombereau 2004). Dulawa et al. (2004) and Dulawa and Hen (2005) used the novelty-induced hypophagia test to study chronic antidepressant effects in mice.

PROCEDURE

For chronic testing, male Balb/cL mice were given various doses of test compound in the drinking water. On day 23 of treatment, mice were singly housed. They were trained to drink sweetened condensed milk for three consecutive days presented for 30 min each day in 10-ml serological pipettes with sippers attached with parafilm. Home cage testing occurred on day 28 when mice were briefly removed from their cages to position pipettes containing milk, and testing began when mice were returned to their home cages. The latency to drink and the volume consumed were recorded every 5 min for 30 min. Home cage testing occurred under dim lighting. Novel cage testing

occurred on day 29, when mice were placed into new clean cages with the same dimensions but without shavings, with pipettes containing the milk positioned. Novel cage testing occurred under bright lighting with white paper under cages to enhance aversiveness. Again, the latency to drink and the volume consumed were recorded every 5 min for 30 min. For latency scores, a maximum cutoff of 600 s was used.

EVALUATION

Two-factor ANOVA with the home/novel cage condition as a within-subjects factor was applied to latency values. For consumption data, a three-factor ANOVA with block (5-min intervals) and the home/novel cage condition as within-subject factors and drug as a between-subject factor was used to assess whether the novel cage was sufficiently anxiogenic to reduce consumption.

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E.6.2.9

Reduction of Submissive Behavior

PURPOSE AND RATIONALE

Pairs of rats competing for food develop a dominant/submissive behavior, which can be measured in a competition test as the relative success of two food-restricted rats to gain access to a sweetened milk supply. Submissive behavior for one subject can be objectively measured as the amount of time spent at the feeder relative to that by the paired dominant animal. Malatynska and Kostowski (1984), Malatynska et al.

(1995, 2002), and Knapp et al. (2002) described reduction of submissive behavior in rats as a test for antidepressant drug activity.

PROCEDURE

Drugs were given by i.p. injection once a day during the treatment period (including weekends). On weekdays, drugs were administered 1 h before behavioral testing. The testing apparatus and the reduction of submissive behavior model procedure are described by Malatynska et al. (2002).

The apparatus was constructed at OmniTech (Columbus, OH) according to a design by Malatynska and Kostowski (1984). The transparent Plexiglas apparatus consisted of two identical chambers ($24 \times 17 \times 14$ cm) connected by a $4.5 \times 4.5 \times 52$ cm passage. In the middle of the passage, a 10-ml glass beaker was placed into a hole cut in the floor of the passage. Prior to behavioral testing, the beaker was filled with sweetened milk (10 g sucrose/cup) through a hinged door in the ceiling of the passage.

The animals were randomly assigned to pairs prior to testing. They were group-housed four to a cage between testing sessions so that paired animals were always separated. Behavioral testing was performed once a day for a 5-min period on weekdays. During the testing period, an animal received one point when it was observed drinking milk during each 5-s interval of the 5-min period. Four different observers contributed to scoring different animal pairs and all were blinded to the treatment animals received. The design of the apparatus permits only one animal to drink at a time but it is possible for both animals to have consumed milk during an interval. At the end of the 5-min period, the animals were returned to their home cages and given free access to food for 1 h. The animals were also given free access to food from the Friday afternoon following testing to the Sunday afternoon when they were once again food-deprived. The animals showed normal weight gain during the course of the study.

EVALUATION

Each animal of the pair was scored on each testing day for 2 weeks. Week 2 data for the two animals of a pair were tested for significant differences using the two-tailed *t*-test assuming unequal variance. The member of a pair having a significantly lower drinking score ($p < 0.05$) was defined as “submissive” and the partner as “dominant.” Any pairs not showing significant dominant–submissive relationship were dropped from the study. Submissive animals were treated with drugs

or vehicle and dominant animals were treated with vehicle for the next 3–4 weeks.

The data used for subsequent analyses are referred to as dominance level values. They are calculated as the difference in daily drinking scores between paired rats. The daily dominance level values were averaged over each week starting with the 2nd week of testing for each pair selected. The *n* used for statistical purposes in the study was the averaged weekly dominance level value for a single pair of animals. The effect of treatment on dominance level values for animal pairs within a treatment group was tested for statistical significance by comparing dominance level values during the 2nd week of testing (control period) to values measured during subsequent weeks of testing (treatment period) by analysis of variance (ANOVA) followed by post hoc unpaired *t*-tests for significance between control and treatment data. A *p*-value of less than 0.05 was used as the cutoff for significance. This approach gives information for the time of effect onset and effect for a selected period. Effect onset is defined as the period (weeks) at which the drug-induced reduction of the dominance level becomes significant relative to the control value measured after the 2nd week before treatment is initiated. The relative effect of two treatments is determined for the same treatment period. This is done for the 3rd week of treatment after all drugs have passed the onset of activity.

MODIFICATIONS OF THE METHOD

Pinhasov et al. (2005) used automatic scoring by a multiple-subject video-tracking system for antidepressant activity testing of the reduction of submission behavior.

Leo et al. (2005) applied nuclear magnetic resonance-based metabonomics to the dominant-submissive rat behavioral model. Principal component analysis revealed a metabolite from milk sugar, galactose, as a discriminating factor between rats classified as dominant and those classified as submissive.

Malatynska et al. (2005) described submissive behavior in **mice** as a test for antidepressant drug activity.

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E.6.2.10

Animal Models of Bipolar Disorder

PURPOSE AND RATIONALE

Bipolar disorder is a psychiatric condition characterized by episodes of mania, depression, and underlying mood instability. The disease is distinguished by its cyclicity. Several attempts have been made to induce cyclic symptoms in animals. Preclinical studies with animal models of mania and depression have been developed to evaluate the potential efficacy of new psychotropic drugs. The main problem is to find a model that mimics mood cyclicity, which is a hallmark of bipolar disorder (Machado-Vieira et al. 2004). Antelman et al. (1995, 1998), Caggiula et al. (1998a, 1998b), and Kucinski et al. (1999) reported that repeated exposure to cocaine or other stressors can induce an oscillation or cycling in various physiological systems.

Cao and Peng (1993) and Arban et al. (2005) described a rodent model of mania, in which hyperactivity is induced by the combination of D-amphetamine and chlordiazepoxide.

PROCEDURE

Male CDI mice weighing 22–26 g were group housed at 21±2°C with 45%–70% humidity on a 12/12h light/dark cycle. Test drugs were administered orally 60 min before the test. D-amphetamine sulfate (1.25 mg/kg i.p.) and chlordiazepoxide (6.25 mg/kg i.p.) were injected 30 min before the session. Locomotor activity was recorded using a Digiscan Analyzer (Model RXYZCM-8, Omnitech). Animals were individually placed in Plexiglas cages equipped with 48

photocells, and the total distance traveled (cm) by each mouse over a 30-min period was determined.

EVALUATION

Statistical analysis was carried out using an ANOVA followed by Dunnett's test. Results are expressed as mean ± SEM of the total distance traveled (in cm), with group size ranging from 9 to 12 mice.

MODIFICATIONS OF THE METHOD

Petty and Sherman (1981) proposed hyperactivity in rats induced by intraventricular injection of 6-hydroxydopamine as a pharmacological model of mania.

Gould et al. (2001) reported differential sensitivity to lithium's reversal of amphetamine-induced open-field activity in two inbred strains of mice.

Gessa et al. (1995) studied sleep deprivation in rats by the platform method as a possible animal model of mania.

Gambarana et al. (2000) found that long-term lithium administration abolishes the resistance to stress in rats sensitized to morphine and discussed this as a model of mania.

Shaldubina et al. (2002) induced a biphasic locomotor response starting with inhibition and followed by excitation by quinpirole, a D₂/D₃ agonist, and recommended this as a model of bipolar disorder.

D'Aquila et al. (2004) treated rats for 4 weeks with imipramine. After a withdrawal time of 40 days, the animals showed a depressive-like behavior indicated by an increase of immobility in the forced swimming test.

El Mallak et al. (1995, 2003) and Decker et al. (2000) administered intracerebroventricularly 5 µl of ouabain (10⁻³ M) to rats, which induced hyper- and hypoactivity and recommended this as a model of bipolar illness.

Wei et al. (2004) generated transgenic mice overexpressing the glucocorticoid receptor specifically in the forebrain. These mice display a significantly increased emotional lability and depressive-like behavior relative to wild type.

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E.6.2.11

Animal Models of Obsessive-Compulsive Disorder

PURPOSE AND RATIONALE

Obsessive-compulsive disorder (OCD) is characterized by recurrent intrusive thoughts, feelings or ideas (obsessions) and ritualistic actions (compulsions) which cause significant distress and dysfunction. The disease has gained increasing attention (Stein 2000, 2002). The relevance of ethologic (naturally occurring) animal models (dogs: Stein et al. 1998; Overall 2000; birds: Woods-Kettelberger et al. 1997; horses: Nurnberg et al. 1997) has been discussed. Clinical studies and pharmacological experiments, mostly performed in rats (Joel et al. 2005; Tsaltas et al. 2005; Joel 2006), indicate the involvement of the serotonergic system.

Deficits in spontaneous alterations produced by 8-OH-DPAT in rats were used as animal model of obsessive-compulsive behavior by Yadin et al. (1991), Fernández-Guasti et al. (2003), van Kuyck et al. (2003), Ulloa et al. (2004a, 2004b), and Agrati et al. (2005).

PROCEDURE

Apparatus

The testing apparatus for spontaneous alterations was a Plexiglas T maze with goal boxes characterized by distinctive cues. All arms (including the main arm and the two goal boxes) measured 50 × 10 cm. Guillotine doors separated the main arm and the goal boxes from the main body of the maze. Small plastic cups were placed in the corners of both goal boxes. The maze was covered with clear Plexiglas lids.

Experimental Procedure

Wistar rats weighing 250–300 g are exposed to three training sessions. On the first training day, animals were habituated to the maze for 20 min during which time they were allowed to explore the entire area. Thereafter, they were exposed to chocolate milk in their home cages, in order to acquaint them to the novel stimulus. On the second training day, animals were confined for 5 min to each goal arm, where chocolate milk was available. On the third training day, animals were placed in the main arm, the guillotine doors were lifted and rats were allowed to choose between the two goal arms, each one baited with chocolate milk. When the animal placed all four paws in a goal arm it was confined to it for 30 s and the entry was considered as a choice. Thereafter, the animals were removed and placed in a holding cage for 10 s. This procedure was repeated for a total nine runs.

Drug treatment (clomipramine or test drugs) was started on three consecutive days before the experimental sessions. One day before, the animals were deprived of food for 24 h.

Thereafter, they were injected i.p. with 1 mg/kg of the selective 5-HT_{1A} agonist 8-OH-DPAT and 15 min later they were placed in the main arm and allowed to choose between the two goal arms. The procedure was repeated up to seven runs or until they alternated their election. The choice made by the animal (right or left) was recorded. The number of repetitive choices until an alternation occurs indicates compulsive-like behavior. Additionally, the animals that chose the same alley in three consecutive occasions were defined as highly perseverative.

EVALUATION

The number of consecutive entries to the same alley of the T-maze is presented as the median and the number of highly perseverative per group is expressed as a percentage. Data were analyzed by means of non-parametric tests.

MODIFICATIONS OF THE METHOD

Joel and Avisar (2001) proposed excessive lever pressing following post-training signal attenuation in rats: a possible animal model of obsessive-compulsive disorder.

Szechtman et al. (2001) discussed compulsive checking behavior of quinpirole-sensitized rats as an animal model of obsessive-compulsive disorder.

Berridge et al. (2005) proposed sequential super-stereotypy of an instinctive fixed action pattern in hyper-dopaminergic mutant mice as a model of obsessive-compulsive disorder.

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E.6.2.12

Antidepressant-Like Activity in Differential-Reinforcement of Low Rate 72-Second Schedule

PURPOSE AND RATIONALE

The differential-reinforcement of low-rate (DRL) 72-s schedule has been recommended for evaluation of antidepressant drugs (O'Donnell and Seiden 1983).

PROCEDURE

Male Sprague Dawley rats weighing 350–450 g are housed in suspended wire cages in rooms maintained at 21–23°C and 30% to 40% relative humidity having free access to laboratory chow except during experimental sessions. Water is provided after each daily session in order to maintain body weights.

Apparatus

Model C Gerbrand operant-conditioning chambers (O'Donnell and Seiden 1983) or Coulbourn operant chambers (Pollard and Howard 1986) are used. A lever that operates a microswitch is mounted on one wall 3 cm from the side, 2.5 cm above a grid floor and 6.5 cm from an access port for a dipper that provides 0.025 ml of water. A houselight is mounted on the opposite wall. A downwards force equivalent to approximately 15 g operates the lever, constituting a response. When the schedule requirements are met, the dipper is lifted from a water trough to an opening in the floor of the access port for 4 s, constituting a reinforcer.

Rats are water-deprived for 22 h before each session. Each rat is initially trained under an alternative fixed-ratio 1, fixed-time 1-min schedule for water reinforcement. Thus, each response is reinforced, and reinforcement is also provided every min if no responding occurs. All rats are then placed under a DRL (differential-reinforcement-of-low-rate) 18 s schedule for 3 weeks. The schedule requirement is then increased to 72 s (DRL 72-sec). When performances on the DRL 72-sec schedule are stabilized (approximately 6 weeks), drug treatments are initiated.

Each group of 6 to 10 rats is subjected to a dose-response determination for one or more drugs. Drugs are administered one h before testing by intraperitoneal injection.

EVALUATION

Data are expressed as percentages of non-injected controls. The control values are the response and reinforcement rates on those days immediately preceding the test day. The number of responses and reinforcers per session at each drug dose are tested for statistically significant differences from control values with paired *t*-tests using a two tailed criterion of statistical significance.

CRITICAL ASSESSMENT OF THE TEST

The method is rather time consuming. Moreover, the specificity as screening method for antidepressants has been challenged (Pollard and Howard 1986).

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E.6.3**Tests for Antidepressant Activity Based on the Mechanism of Action****E.6.3.1****Potential of Norepinephrine Toxicity****PURPOSE AND RATIONALE**

Antidepressants block the re-uptake of biogenic amines into nervous tissue. In this way, the toxic effects of norepinephrine are potentiated.

PROCEDURE

Male NMRI mice (22–25 g) are randomly assigned to test groups of 10 subjects. The test drug, the standard or the vehicle are given orally 1 h prior to the s.c. injection of the sublethal dose of 3 mg/kg noradrenaline. The groups of 10 mice are placed into plastic cages with free access to food and water.

EVALUATION

The mortality rate is assessed 48 h post-dosing. *ED*₅₀, or dose which causes death of 50% of the treated subjects, is calculated by means of linear regression analysis.

CRITICAL ASSESSMENT OF THE METHOD

Several antidepressants block not only the uptake of noradrenaline, but also of dopamine and of serotonin.

By definition, this test can only measure the noradrenaline-uptake inhibition.

One of the earliest observations on the pharmacology of antidepressants was by Sigg (1959), who showed that imipramine, as distinguished from other tricyclic substances known at this time, produced a profound potentiation of the cardiovascular effects of exogenously administered catecholamines in animals.

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E.6.3.2

Compulsive Gnawing in Mice

PURPOSE AND RATIONALE

In man and in other species, like dogs, apomorphine induces emesis. Treatment of rodents with apomorphine causes compulsive gnawing instead of vomiting. The compulsive gnawing in mice induced by apomorphine is due to dopaminergic stimulation. Centrally acting anticholinergics shift the balance between acetylcholine and dopamine resulting in an enhancement of the apomorphine effect. Therefore, many compounds with psychotropic activity are known to have an apomorphine-synergistic effect. This enhancement is also found after administration of tricyclic antidepressants (Ther and Schremm 1962).

PROCEDURE

NMRI mice with a body weight between 18 and 20 g are injected s.c. with 10 mg/kg apomorphine. At the same time they are treated i.p. or s.c. with the test drug or the vehicle. For testing oral activity the animals are treated 30 min prior to apomorphine injection. Immediately after apomorphine injection 6 mice are placed into a cage 45 × 45 × 20 cm with a wired lid. The bottom of the cage is covered with corrugated paper, the corrugation facing upwards. The mice start to bite into the paper causing fine holes or tear the paper. This behavior is enhanced by antidepressants. The mice remain 1 h in the cage.

EVALUATION

The number of bites into the corrugated paper is evaluated by placing a template upon the paper. The template has 10 rectangle windows divided into 10 areas of the same size. In a total of 100 areas the number

of bites is checked. In this way percentage of damaged paper is calculated. Ten mg/kg apomorphine does not increase the biting rate of 5–10% which also occurs in normal animals. Likewise, atropine in doses of 40 and 80 mg/kg s.c. alone does not increase the biting behavior. In contrast, the combination of apomorphine and atropine greatly enhances the occurrence of gnawing. The same is true for antidepressants. For example, a dose of 25 mg/kg imipramine increases the damaged area by 40–70%. Percent gnawing of the test compound is compared with that of the standard.

CRITICAL ASSESSMENT OF THE TEST

Not only antidepressants, but also centrally acting anticholinergics and antihistaminics are active in this test. However, the test has the advantage of simplicity without any pretraining of the animals.

MODIFICATIONS OF THE METHOD

De Feo et al. (1983) reported on possible dopaminergic involvement in biting compulsion of mice induced by large doses of clonidine.

Stereotyped gnawing in mice is induced by methylphenidate (Pedersen and Christensen 1972). The phenomenon is potentiated by various drugs, such as benzodiazepines (Nielsen et al. 1991).

Stereotyped behavior in **guinea pigs** induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

Molander and Randrup (1974) investigated the mechanisms by which L-DOPA induces gnawing in mice.

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E.6.3.3

Apomorphine-Induced Hypothermia in Mice

PURPOSE AND RATIONALE

Apomorphine induces hypothermia in mice which can be prevented by antidepressants.

PROCEDURE

Male NMRI mice (20–22 g) are used and randomly assigned to test groups of 6 subjects. One hour after oral administration of the test compounds or the vehicle a dose of 16 mg/kg apomorphine is injected s.c. The rectal temperature of each mouse is measured by an electronic thermometer immediately prior to apomorphine administration and 10, 20 and 30 min later. During the entire experiment, subjects are housed in groups of three in glass jars at room temperature.

EVALUATION

A time curve is constructed by plotting the temperature (mean of each group) against time in min. The AUC is calculated for all groups and converted into percent inhibition of apomorphine-induced hypothermia in the control group. An ED_{50} can be calculated by linear regression analysis.

CRITICAL ASSESSMENT OF THE METHOD

Antagonism against apomorphine-induced hypothermia can be regarded as a hint for antidepressant activity. Compounds with a marked noradrenergic or dopaminergic component are active against apomorphine-induced hypothermia but not antidepressants acting mainly through serotonergic mechanisms.

MODIFICATIONS OF THE METHOD

Cox and Lee (1981) induced hypothermia in rats by intrahypothalamic injection of 5-hydroxytryptamine and recommended this as a model for the quantitative study of 5-hydroxytryptamine receptors.

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E.6.3.4

Tetrabenazine Antagonism in Mice

PURPOSE AND RATIONALE

Tetrabenazine (TBZ) induces a depletion of biogenic amines (e.g. noradrenaline, dopamine, serotonin) without affecting their *de novo* synthesis. TBZ depletes noradrenaline from nerve terminals and prolongs re-uptake into the granula. Noradrenaline is degraded by monoamino-oxidase. Antidepressants inhibit the re-uptake of noradrenaline into the nerve terminals and increase thereby the noradrenaline concentration at the receptor site. In this way, the effect of TBZ is antagonized. Therefore, both MAO-inhibitors and tricyclic antidepressants are known to prevent or to antagonize these effects. The prevention of TBZ induced ptosis and catalepsy can be used for evaluation of antidepressants.

PROCEDURE

Groups of 5–10 male NMRI mice (20–22 g) are used. Sixty min after oral or 30 min after i.p administration of the test compound or the vehicle 40 mg/kg i.p. TBZ are injected. The animals are placed into individual cages. The test is started 30 min after TBZ administration and repeated every 30 min up to 2 h. Catalepsy and ptosis are used as criteria. A stair is formed with 2 cork stoppers having 2 steps of 3 cm height. The animals are placed head downwards with their hindlegs upon the top cork. As long as TBZ exerts its cataleptic effect the animals remain in this catatonic state. If the cataleptic effect is not antagonized after a limit of 60 s the animals are placed into a normal position.

EVALUATION

Thirty days after replacement the degree of ptosis is scored: eyes closed=4, eyes 3/4 closed=3, eyes 1/2 closed=2, eyes 1/4 closed=1, eyes open=0. (Rubin et al. 1957) Similarly, the cataleptic effect is scored according to the duration of catalepsy. Catalepsy more than 60 s=5, between 30 and 60 s=4, between 10 and 30 s=3, between 5 and 10 s=1, less than 5 s=0. The scores of the TBZ controls are taken as 100% and the percentage is calculated for the treated animals.

Imipramine was found to be effective at a dose of 10 mg/kg s.c. or 20 mg/kg orally.

CRITICAL ASSESSMENT OF THE METHOD

The TBZ antagonism has been found to be a simple and reliable test for evaluation of classical antidepressants.

MODIFICATIONS OF THE METHOD

Ptosis in mice can be induced in a similar way by treatment with reserpine. Mice receive a single oral dose of the test compound followed by subcutaneous administration of 5 mg/kg reserpine 30 min later. Ptosis is observed for the next 1 or 2 h and scored in a similar way as in the TBZ test.

TBZ ptosis can also be elicited in rats. The test procedure and the evaluation is the same as in mice. Tetra-benzazine-induced hyperthermia in mice has been proposed as test for antidepressant activity by Gylys et al. (1963).

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E.6.3.5

Reserpine-Induced Hypothermia

PURPOSE AND RATIONALE

Depletion of biogenic amines (noradrenaline, 5-hydroxytryptamine, dopamine) in the brain induces not only catalepsy and ptosis but also hypothermia in rodents. The decrease of body temperature induced by

reserpine is antagonized by antidepressants, MAO-inhibitors and central stimulants. The subcutaneous administration of 2 mg/kg reserpine leads to a decrease of core temperature in mice to 20–23°C after 18 h. The fall in temperature can be antagonized by antidepressants, but also by amphetamine-like drugs. However, the time course is different: tricyclic antidepressants have a slow onset of action and a long lasting effect, whereas amphetamine-like drugs have a quick onset of action and a short-lasting effect.

PROCEDURE

Groups of 5 male NMRI mice (19–21 g body weight) are used. On the day before testing, they are dosed with 2 mg/kg reserpine s.c. They are housed in a climate-controlled animal colony and have free access to food and water. Eighteen hours after reserpine administration, the animals are placed into individual cages. The initial rectal temperature is determined by insertion of an electronic thermometer (e.g. Ellab T-3) to a constant depth of 2 cm. Following administration of the test compound (either i.p. or p.o.), the rectal temperature is measured again at 60 min intervals for 7 h.

EVALUATION

Rectal temperature is recorded every hour. The difference in temperature from vehicle controls is calculated for each time and the maximal difference is scored. The differences are then statistically compared using the *t*-test.

MODIFICATIONS OF THE METHOD

The time course and the administration route can be changed. Male mice are treated with the test drug or the standard 1 h prior to intravenous injection of 2 mg/kg reserpine. Rectal temperature is measured by a rectal thermometer prior and every 60 min up to 6 h. The animals are kept in groups of 3 in glass jars at a controlled temperature of 20–21°C. Using a computer program the area under the curve is calculated by plotting the temperature (mean of each group) before and the decrease after reserpine against time in hours. Areas of treated groups are converted to percentage of controls.

Colpaert et al. (1975), Niemegeers (1975) described the antagonism of antidepressants and other drugs against Ro-4–1284, a benzoquinolizine derivative which by itself exhibits reserpine-like activities.

CRITICAL ASSESSMENT OF THE METHOD

The test has been proven as a simple and reliable method to detect antidepressant activity. However, the

reversal of hypothermia is not specific for antidepressants. The fall in body temperature can also be antagonized by amphetamines, and some antipsychotic agents (chlorpromazine). The different time course of antidepressants (slow onset of action, long-lasting effect) and amphetamine-like drugs (quick onset of action, short-lasting effect) allows differentiation between the two groups of drugs.

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E.6.3.6

5-Hydroxytryptophan Potentiation in Mice

PURPOSE AND RATIONALE

According to the monoamine hypothesis of depression compounds exert antidepressant activity because they are capable of enhancing central noradrenergic and/or serotonergic functions. Several antidepressant agents potentiate serotonin effects by a block of the re-uptake of serotonin. DL-5-Hydroxytryptophan is used as the precursor of serotonin. Enzymatic breakdown is inhibited

by the MAO-inhibitor pargyline. In mice the characteristic symptom of head-twitches is observed.

PROCEDURE

Groups of 6 male mice (18–30 g) are used. They are treated i.p with the test drug or the vehicle. Thirty min later, the mice receive 75 mg/kg pargyline HCl s.c. Ninety min after pargyline the animals are injected with 10 mg/kg DL-5-hydroxytryptophan (5-HTP) i.v.

EVALUATION

Animals positively influenced show a characteristic behavior of head twitches. A animal is considered to be positive if it shows head twitches 15 min after 5-HTP injection. Enhancement is observed after treatment with serotonin uptake blockers relative to animals pretreated with pargyline only.

CRITICAL ASSESSMENT OF THE METHOD

The test can be considered as additional evidence for antidepressant activity based on uptake inhibition.

MODIFICATION OF THE METHOD

The head-twitch in mice can also be elicited without a MAO-inhibitor by using higher doses (200 mg/kg) of DL-5-hydroxytryptophan.

Moser and Redfern (1988) studied the effects of four benzodiazepines on the head-twitch response induced in mice by several 5-HT receptor agonists.

Meert et al. (1988) studied partial and complete blockade of 5-hydroxytryptophan (5-HTP)-induced head twitches in the *rat* by serotonin S_2 antagonists.

Niemegeers et al. (1983), Awouters et al. (1988) used *mescaline* induced head twitches in the *rat* as an *in vivo* method to evaluate serotonin S_2 -antagonists. Head twitches were counted for 15 min after intravenous injection of 20 mg/kg mescaline. Twitch counts of less than 2 were considered as inhibition and of less than 2 as blockade of the mescaline response.

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E.6.3.7

5-Hydroxytryptophan Potentiation in Rats

PURPOSE AND RATIONALE

The inhibition of serotonin re-uptake by some antidepressants can be tested *in vivo* in rats by administration of the precursor 5-hydroxytryptophan and inhibition of its breakdown by the MAO-inhibitor pargyline. In contrast to mice exhibiting head twitches, rats show other symptoms such as continuous forelimb clonus.

PROCEDURE

Groups of 6 male Wistar rats weighing 150–200 g are used. Four hours prior to testing pargyline HCl is injected sc. at a dose of 75 mg/kg. Thirty min before i.p. injection of 1 mg/kg L-5-hydroxytryptophan, test compounds or standards are administered intraperitoneally. Fifteen min after the 5-hydroxytryptophan injection, the animals are observed for 15 min. An animal is considered to be positive if it exhibits continuous forelimb clonus.

EVALUATION

Enhancement is expressed as normalized percent increase relative to the vehicle control. Using various doses, ED_{50} values with 95% confidence limits can be determined by probit analysis.

MODIFICATIONS OF THE METHOD

Hallberg et al. (1985) developed a registration device based on accelerometry in order to accomplish an objective quantification of tremors in conscious unrestrained rats. Tremor intensity was continuously recorded by a small piezoresistive accelerometer mounted on the back of the freely moving rat. The accelerometer was connected to a Grass Polygraph. The integrated signals were further analyzed by a desk-top computer.

The behavior in rats induced by 10 mg/kg L-5-hydroxytryptophan i.p. can be antagonized by compounds having 5-HT₂ antagonist properties (Colpaert and Janssen 1983).

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E.6.3.8

Yohimbine Toxicity Enhancement

PURPOSE AND RATIONALE

Yohimbine occupies central α_2 -receptors, and prevents noradrenaline from binding to these receptors. Compounds with antidepressant properties are known to inhibit physiological inactivation of noradrenaline and other biogenic amines by blocking the re-uptake at nerve terminals. Administration of a test compound with antidepressant properties leads to an increase in noradrenaline concentration. Following the simultaneous administration of yohimbine and antidepressants the animals die of noradrenaline poisoning.

PROCEDURE

Groups of 10 male NMRI mice (25–28 g) are used. Mice are placed in plastic cages and receive the test compound or the vehicle by oral or i.p. administration.

Thirty min later, a dose of 25 mg/kg yohimbine (a sublethal dose) is given s.c.

EVALUATION

Mortality rate is assessed 1 h, 2 h, 3 h, 4 h, 5 h, and 24 h after dosing. Lethality in the control group (Yohimbine only) is less than 10%, whereas 10 mg/kg desipramine-HCl causes death in about 90%. Using various doses ED_{50} -values can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The test has been proven as a simple method to detect antidepressants with monoamine uptake inhibiting properties.

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E.6.3.9

Tryptamine Seizure Potentiation in Rats

PURPOSE AND RATIONALE

Monoamine oxidase (MAO) inhibitors like iproniazid enhance seizures in rats caused by an intravenous infusion of tryptamine HCl. This procedure can be used to elucidate the *in vivo* MAO inhibiting properties of compounds.

PROCEDURE

Groups of 5 male Wistar rats weighing 150–200 g are used. Test compounds, standard or vehicle controls are administered intraperitoneally 0.5; 1; 2; and 4 h prior testing. At the time of testing 5 mg/kg tryptamine HCl freshly dissolved in saline are injected intravenously. Immediately after tryptamine HCl adminis-

tration, the animals are observed individually for three min for the appearance of clonic “pedalling” movements of the forepaws which is considered a positive response. Frequently, these clonic seizures are preceded by a kyphotic curvature of the spine but this sign does not constitute a positive response.

In addition to the vehicle control group, a series of five positive control animals receiving tranylcypromine at 5 mg/kg i.p. with a 0.5 h pretreatment time are subjected to the test in order to check the effectiveness of the tryptamine HCl solution which is relatively unstable. A 100% response is expected. Fresh tryptamine HCl solution should be prepared hourly as needed.

EVALUATION

The normalized percent potentiation is calculated as follows:

$$100 \times \left[\frac{\frac{\% \text{ animals potent. in drug group}}{1 - \% \text{ animals potent. in vehicle group}}}{\frac{\% \text{ animals potent. in vehicle}}{1 - \% \text{ animals potent. in vehicle group}}} \right]$$

A dose-response is obtained in the same manner at the peak time of drug effect except that a group size of 10 animals is used and four different doses are tested in addition to the vehicle and the tranylcypromine groups.

An ED_{50} is calculated using probit analysis.

MODIFICATIONS OF THE METHOD

Graham-Smith (1971) described the inhibitory effect of chlorpromazine on the syndrome of hyperactivity produced by L-tryptophan or 5-methoxy-N,N-dimethyltryptamine in rats treated with a monoamine oxidase inhibitor.

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E.6.3.10**Serotonin Syndrome in Rats****PURPOSE AND RATIONALE**

Compounds which stimulate serotonin receptors cause a series of behavioral changes in rats which is called the serotonin syndrome (Jacobs 1976; Green and Heal 1985; Tricklebank 1985) such as head weaving, increased locomotion, forepaw treading, flat posture and lower lip retraction. With increasing knowledge about the subtypes of serotonin receptors these symptoms were defined to be associated with 5-HT_{1A} receptors and their specific agonists (Smith and Peroutka (1986), Blanchard et al. 1993; Yu and Lewander 1997).

PROCEDURE

Time- and dose-dependence of **forepaw treading**, which is scored at 15, 30, 45, and 60 min according to a 3-point scale (0 = absent; 1 = periodic and weak; 2 = continuous) according to Arnt and Hyttel (1989) and Porsolt et al. (1992),

was measured by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Berendsen and Broekkamp (1990), Schoeffter et al. (1993), Andersson and Larsson (1994), Forster et al. (1995), Kofman and Levin (1995), Lu and Nagayama (1996), O'Connell and Curzon (1966), Gaggi et al. (1997), Kleven et al. (1997), Yu and Lewander (1997),

of **flat body posture** by Deakin and Green (1978), Goodwin and Green (1985), Goodwin et al. (1986), Blanchard et al. (1993), Schoeffter et al. (1993), Andersson and Larsson (1994), Foreman et al. (1993, 1994, 1995), Forster et al. (1995), Kofman and Levin (1995), O'Connell and Curzon (1966), Gaggi et al. (1997), Kleven et al. (1997), Wolff et al. (1997), Yu and Lewander (1997),

of **hind limb abduction** by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Kofman and Levin (1995),

of **increased motility** by Tricklebank (1985), Forster et al. (1995), Gaggi et al. (1997), O'Neill and Parameswaran (1997),

of **decreased body temperature** by Martin et al. (1992), Schoeffter et al. (1993), Simiand et al. (1993), Foreman et al. (1993, 1994, 1995), Forster et al. (1995), O'Connell and Curzon (1966), Bagdy and To (1997), Wolff et al. (1997), Yu and Lewander (1997),

of **head twitches** by Deakin and Green (1978), Green et al. (1983), Goodwin and Green (1985), Goodwin et al. (1986), Meert et al. (1988), Berendsen and

Broekkamp (1990), Kofman and Levin (1995), Gaggi et al. (1997), Kleven et al. (1997),

of **lower lip retraction**, which is scored according to Berendsen et al. (1989) after 15, 30, 45 min as follows: 0 = lower incisors not or hardly visible (not different from nontreated animals), 0.5 = partly visible, 1 = completely visible,

by Smith and Peroutka (1986), Berendsen et al. (1994, 1997), Porsolt et al. (1992), Blanchard et al. (1993), Andersson and Larsson (1994), Foreman et al. (1993, 1994, 1995), Moore et al. (1993), De Boer et al. (1995), Berendsen et al. (1966), Bagdy and To (1997), Groenink et al. 1997; Kleven et al. (1997), Wolff et al. (1997).

EVALUATION

Scores of each symptom are registered for each test animal. Average values of treated animals are compared with controls treated with vehicle alone. Dose-response and time response curves are evaluated.

CRITICAL ASSESSMENT OF THE TEST

Among the many symptoms of the serotonin syndrome in rats, forepaw treading, flat body posture and lower lip retraction were used by most authors to characterize agonists and antagonists of 5-HT_{1A} receptors.

MODIFICATIONS OF THE METHOD

Trulson et al. (1976) used the serotonin syndrome to test the supersensitivity after destruction of central serotonergic nerve terminals by intracerebral injection of 5,7-dihydroxytryptamine.

Blanchard et al. (1997) described the symptoms of selective activation of 5-HT_{1A} receptors in **mice**.

Locomotor activity of **guinea pigs** was increased in a dose-dependent manner by 5-HT_{1A} receptor agonists (Evdenden 1994).

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E.6.3.11

Hypermotility in Olfactory-Bulbectomized Rats

PURPOSE AND RATIONALE

Bilateral olfactory bulbectomy in the rat is associated with changes in exploratory behavior that are reversed by chronic, but not acute treatment with antidepressant drugs (Cairncross et al. 1978, 1979; Leonard and Tuite 1981; Janscár and Leonard 1981). The olfactory bulbectomized rat is used as an animal model of depression. Several behavioral changes have been observed following bilateral olfactory bulbectomy: hyperactivity in a closed arena, such as the open field; enhanced nocturnal activity; deficits in memory as shown by passive avoidance behavior and in the Morris water maze and the radial maze; increased open-arm entries in the elevated-plus maze, and changes in food-motivated and conditioned taste aversion behavior. Alteration in the noradrenergic, serotonergic, cholinergic, GABAergic, and glutamatergic neurotransmitter systems are also associated with olfactory bulbectomy (Wren et al. 1977; Van Riezen and Leonard 1990; Kelly et al. 1997).

PROCEDURE

Male Sprague Dawley rats are anesthetized with intraperitoneal injection of 2.5% tribromo-ethanol solution (Cairncross et al. 1977). Following exposure of the skull, a burr hole is drilled at points 7 mm anterior to the bregma and 2 mm either side of the midline at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs are removed by suction and the burr holes filled with hemostatic sponge. Tetramycin powder is then applied to the wound and the skin closed by surgical clips. Sham-operated animals are treated in the same way but although the dura above the bulbs is cut, the bulbs are left intact. The animals are allowed to recover for 14 days after surgery.

For drug evaluation the animals are treated subcutaneously with the test drug or the standard or the vehicle once daily at 09:00 for 14 days. In each experiment sham-operated controls treated with test drug, standard and vehicle are included.

The behavior of the animals is tested from the 12th day onwards. The rats are placed singly in the center of an open field apparatus. Ambulation (no. of squares crossed), rearing (forepaws raised from the

floor), grooming and defecation (no. of fecal boli) scores are recorded for an 3 min period of observation.

EVALUATION

The results are analyzed statistically using Student's *t*-test (2 tail test), the significance being set at $P < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

The bulbectomized rat model has been shown to be highly selective for both typical and atypical antidepressants, however, the procedure is quite time consuming.

MODIFICATIONS OF THE METHOD

Various authors used this model to demonstrate antidepressant-like activity: such as Briley et al. (1996) for milnacipran, a serotonin and noradrenaline uptake inhibitor, Hancock et al. (1995) for A-80426, an α_2 -adrenoceptor antagonist with serotonin uptake blocking activity, McNamara et al. (1995) for the centrally active serotonin agonist 8-hydroxy-2-(di-n-propylamino) tetrinalin (8-OH-DPAT), Redmont et al. (1997) for dizocilpine (MK-801), Song and Leonard (1994) for the serotonin reuptake inhibitors fluvoxamine and sertraline, Song et al. (1996a) for centrally administered neuropeptide Y, Song et al. (1996b) for the H_1 receptor antagonist terfenadine.

Kelly and Leonard (1994, 1995) studied the effects of potential antidepressants, such as selective serotonin reuptake inhibitors, in olfactory bulbectomized rats. Kelly et al. (1997) anesthetized male Sprague Dawley rats weighing 230–280 g with 375 mg/kg i.p. chloral hydrate. The surgical procedure involves drilling two burr holes (2 mm diameter, 8 mm anterior to bregma) either side, 2 mm from the midline of the frontal bone overlying the olfactory bulbs. The olfactory bulbs can be visualized through these burr holes and can be aspirated by means of a blunt hypodermic needle attached to a water pump, taking care not to cause damage to the frontal cortex. Prevention of blood loss from the burr holes is achieved by filling them with hemostatic sponge. Antibiotic powder is applied to the wound, prior to closing with 7.5-mm surgical clips. A period of 2 weeks allows for the recovery from the surgical procedure and is optimal for the development of the bulbectomy syndrome.

Potential antidepressant drugs are preferably administered for a period of 2 weeks. Various procedures can be used to test the drug's effect in olfactory bulbectomized rats, such as open field (Cryan et al. 1999; Slotkin et al. 1999), home cage activity (Redmont et al.

1997), forced swim test (Kelly and Leonard 1999), elevated plus maze (McGrath and Norman 1999), passive avoidance (Martin et al. 1998; Nowak et al. 2003), and olfactory bulbectomy-induced hyperemotionality (Chaki et al. 2004).

Redmont et al. (1995) studied the effect of chronic antidepressant administration on the conditioned taste aversion to 8-OHDPAT in the olfactory bulbectomized rat.

Kelly et al. (1997) gave an update of the olfactory bulbectomized rat as a model of depression. Tricyclic antidepressants (amitriptyline, desimipramine), atypical agents (mianserin), selective serotonin reuptake inhibitors (paroxetine, sertraline, fluvoxamine), reversible inhibitors of monoamine oxidase A (moclobemide), as well as putative antidepressants, such as 5-HT_{1A} agonists (zalospirone, ipsapirone), noncompetitive NMDA antagonists (MK-801) and triazolobenzazepines (alprazolam, adinazolam), have demonstrated antidepressant-like activity in this model.

Zhou et al. (1998) found serotonergic hyperinnervation of the frontal cortex in the bulbectomized rat.

Increases in the concentrations of the neuropeptides corticotropin-releasing factor, thyrotropin-releasing factor, somatostatin (Bisette 2001), and neuropeptide Y (Holmes et al. 1998), which may play a role in mediating the antidepressant-sensitive behaviors, have been demonstrated.

Comparing the behavioral and biochemical effects of bulbectomy in young versus aged rats, Slotkin et al. (1999) suggested that this test might provide a useful animal model with which to test therapeutic intervention for geriatric depression.

Ho et al. (2000) demonstrated increased striatal glutamate release during novelty exposure-induced hyperactivity in olfactory bulbectomized rats that may have a modulatory role in the antidepressant-sensitive response.

Wrynn et al. (2000) performed an *in vivo* magnetic resonance imaging study in olfactory bulbectomized rats and demonstrated alterations in signal intensities in cortical, hippocampal, caudate and amygdaloid regions.

Holmes et al. (1998) studied the effects of olfactory bulbectomy on neuropeptide gene expression in the rat olfactory system.

Bilkei-Gorzo et al. (2002) and Zueger et al. (2005) tested alterations in locomotor activity and exploratory behavior after olfactory bulbectomy in mice (using systems provided by TSE Systems, Bad Homburg, Germany).

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E.6.3.12

Sexual Behavior in Male Rats

PURPOSE AND RATIONALE

Sexual behavior in male rats is stimulated by 5-HT_{1A} receptors agonists (Ahlenius et al. 1981; Gorzalka et al. (1990), Foreman et al. 1993, 1994, 1995; Anderson and Larsson 1994; Tallentire et al. 1996; Ahlenius and Larsson 1997; Fernández-Guasti and Rodríguez-Manzo 1997) and inhibited by serotonin receptor antagonists (Mendelson and Gorzalka 1981) and by 5-HT_{1B} receptors antagonists (Fernández-Guasti et al. 1989). The test procedure was described in detail by Arnone et al. (1995).

PROCEDURE

Adult male and ovariectomized female Sprague Dawley rats are housed in sex-separated rooms at 21 ± 1°C in a reversed light-dark cycle with free access to food and water.

Mating Behavior

Male rats of two levels of sexual performance are selected for drug testing: sexually naive and sexually experienced. The latter are given 4 mating pretests, twice a week. Only sexually active males that achieve at least two ejaculations per test, are included in the experiments.

The mating tests are performed between 12:30 and 17:00, during the dark phase of the lighting cycle. Drug is administered orally 1 h before the test to the male rat. The animal is allowed to adapt to the test area (60 cm diameter, 50 cm high) illuminated with a dim red light. Each behavioral test starts with the introduction of a stimulus female brought into sexual receptivity by s.c. treatment with estradiol benzoate (10 µg/rat), followed 48 h later by progesterone (500 µg/rat), 4–5 h before testing. The tests end either 20 min later or after the first post-ejaculatory mount (or 2 h later for sexual satiation).

The following behavioral parameters are recorded:

- **Mount and intromission latencies:** time interval from the first introduction of the female to the first mount or intromission, respectively. Mounts are accompanied by an average of three or four brief shallow thrusts, while the intromission, which succeeds

this event, is marked by a single deep thrust indicative of penile insertion.

- **Mount frequency:** total number of pre-ejaculatory mounts with and without intromission.
- **Intromission frequency:** total number of pre-ejaculatory intromissions.
- **Ejaculatory latency:** time interval from the first intromission to ejaculation. A total of 1200 s is scored for the latencies of rats failing to mount, intromit or ejaculate.

Sexual Satiation

The mating pattern of the male rat consists of repeated mounts and intromissions, culminating in an ejaculation. The ejaculation is followed by a period of 4–5 min during which time the male remains refractory to sexual stimulation by the female. The sexual activity is thereafter resumed with a new series of mounts and intromissions followed by ejaculation. If uninterrupted, the rat may achieve five to six ejaculations before sexual satiation sets in. Sexually experienced male rats are allowed to copulated with a receptive female for 2 h. The behaviors are recorded on a videotape. The criterion for satiation is that the male fails to mount within 20 min post ejaculation. The latency to satiation and the number ejaculations are recorded.

Finally, for each ejaculatory series, the following parameters are recorded:

- **Copulation duration:** time interval between the first mount and the ejaculation.
- **Post ejaculatory interval:** time interval from the ejaculation to the first mount of the next series.

Penile Erections

One hour before the experiment, male rats are allowed to adapt to the quiet observation room. One hour after oral drug administration, rats are placed in individual plastic cages (10.5 × 24 × 16 cm). Series of nine rats comprising, at random, control and drug-treated animals, are observed simultaneously for 45 min and the number of penile erections is counted. Penile erection is defined as a period of pelvic thrusting followed by an upright position with genital grooming and the display of the engorged penis. Animals are used only once.

EVALUATION

Data are analyzed using non-parametric statistics. The Fisher test is used for percentage responding. For

quantified behavioral parameters, the Kruskal-Wallis test, followed by the Mann-Whitney *U*-test corrected by Holm's method is used for comparisons versus the control group. The Mann-Whitney *U*-test is used for comparison of a single treated group versus its own control group.

MODIFICATIONS OF THE METHOD

Barr et al. (1999) subjected male rats to a 4-day escalating dose schedule of d-amphetamine. Twelve hours after the final dose the rats were tested for sexual behavior. Withdrawal from the amphetamine was associated with decrements in several motivational components of sexual behavior. The procedure was recommended as a rodent model of depression.

Pomerantz et al. (1993) studied the influence of 5-HT receptor agonists on male sexual behavior of **rhesus monkeys**.

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E.6.4

Genetic Models of Depression

E.6.4.1

Flinders Sensitive Line of Rats

The **Flinders Sensitive Line of rats** was established by genetically selecting (breeding) Sprague Dawley rats for a behavioral trait, supersensitivity to cholinergic agents (Overstreet and Russell 1982; Overstreet 1986; Overstreet et al. 1986, 1990, 1992; Daws and Overstreet 1999). The name Flinders refers to University of Flinders, Australia, where the line was first selected. The usefulness of the Flinders Sensitive Line of rats as an animal model for depression is evident because it exhibits behavioral features characteristic of depression in humans and responds to chronic, but not acute, treatment with antidepressants (Overstreet 1993; Overstreet et al. 1995; Yadid et al. 2000; Overstreet and Griebel 2004; Overstreet et al. 2004; Dremencov et al. 2005).

Vasquez et al. (2000) tested the effects of electroconvulsive stimuli and d-amphetamine on neuropeptide Y concentrations in brain and on locomotion in the Flinders Sensitive Line rat.

Ferreira-Nuño et al. (2002) studied masculine sexual behavior features in Flinders sensitive and resistant line rats.

Shayit et al. (2003) demonstrated 5-HT_{1A} receptor subsensitivity in infancy and supersensitivity in adulthood in the Flinders Sensitive Line rat.

Dremencov et al. (2004) showed in the Flinders Sensitive Line of rats that the serotonin–dopamine interaction is critical for the fast-onset action of antidepressant treatment. Seven-day treatment with nefazodone (a putative fast-onset antidepressant) but not with desipramine (a classical antidepressant) normalized immobility time in the swim test in Flinders Sen-

sitive Line rats. Serotonin-induced dopamine release but not basal dopamine levels correlated with the improvement of depressive-like behavior.

Lavi-Avnon et al. (2005) showed an abnormal pattern of maternal behavior in the Flinders Sensitive Line of rats.

Maayan et al. (2005) demonstrated the involvement of dehydroepiandrosterone and its sulfate ester in blocking the therapeutic effect of electroconvulsive shocks in the Flinders Sensitive Line of rats.

King and Edwards (1999), King et al. (2001), and Shumake et al. (2003) bred **congenital learned helpless rats**, which exhibit physiological symptoms of analgesia, cognitive deficits and hyporesponsivity of the hypothalamic–pituitary–adrenal axis similar to those observed in human subjects with post-traumatic stress disorder.

Will et al. (2003) described **selectively bred Wistar–Kyoto rats** as an animal model of depression and hyper-responsiveness to antidepressants. Based on the significant decrease in investigative behavior of male rats towards a female intruder, reflecting the presence of anhedonia, Paré (2000) concluded that the Wistar–Kyoto rat strain represents a useful animal model of depressive behavior.

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- Cases et al. (1995) found aggressive behavior and altered amounts of brain serotonin and epinephrine in mice lacking MAO_A. Pup behavioral alterations including trembling, difficulties in righting, and fearfulness were reversed by the serotonin inhibitor parachlorophenylalanine. Adults manifested a distinct behavioral syndrome, including enhanced aggression in males.
- Lijam et al. (1997) created mice deficient in *Dvl1*, one of the three mouse homologs of the *Drosophila* polarity gene *Dishevelled*, by gene targeting. These mice exhibited reduced social interaction and sensorimotor gating abnormalities.
- Grimsby et al. (1997) reported an increased stress response and increased levels of α -phenylethylamine in MAO_B-deficient mice. In addition, mutant mice were resistant to the neurodegenerative effects of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), a toxin that induces a condition reminiscent of Parkinson's disease.
- Sallinen et al. (1999) reported that general alteration of the α_2 -adrenoceptor subtype c in mice affects the development of behavioral despair and stress-induced increases in plasma corticosterone levels. α_{2c} -Adrenoceptor overexpression increased, and the lack of α_{2c} -adrenoceptors, decreased the immobility of mice in the forced swimming test.
- Cyran et al. (2001) used **dopamine-beta-hydroxylase-deficient** mice to determine the role of norepinephrine in the mechanisms of action of antidepressant drugs. The dopamine-beta-hydroxylase-deficient mice failed to demonstrate antidepressant-like behavioral effects following the administration of several classes of antidepressants.
- Mayorga et al. (2001) studied antidepressant-like behavioral effects in **5-hydroxytryptamine_{1A} and 5-hydroxytryptamine_{1B} receptor mutant mice**. The results suggested, that 5-HT_{1A} and 5-HT_{1B} receptors have different roles in the response to antidepressant drugs in the tail-suspension test.
- Froger et al. (2001) found that 5-hydroxytryptamine_{1A} autoreceptor adaptive changes in **substance P (neurokinin 1) receptor knockout mice** mimic antidepressant-induced desensitization. The constitutive lack of NK₁ receptors appeared to be associated with a downregulation/functional desensitization of 5-HT_{1A} autoreceptors resembling that induced by chronic treatment with antidepressants.
- Jaber et al. (1997) inactivated the expression of the **dopamine transporter** in mice. The authors claimed that these genetically altered mice offer a unique model to test the specificity and selectivity

E.6.4.2 Genetically Altered Mice as Models of Depression

Cryan et al. (2002) published a survey on the use of genetically altered mice to assess antidepressant-related phenotypes. Cyran and Mombereau (2004) discussed the utility of models for studying depression-related behavior in genetically modified mice.

of dopamine-transporter-acting drugs and provide new concepts related to clinical conditions such as Parkinson's disease, schizophrenia, and drug addiction.

Xu et al. (2000) reported that mice lacking the **norepinephrine transporter** are supersensitive to psychostimulants. Disruption of the norepinephrine transporter (NET) gene prolonged the clearance of norepinephrine and elevated extracellular levels of this catecholamine. In a classical test for antidepressant drugs, the NET-gene-deficient mice behaved like antidepressant-treated wild-type mice.

Holmes et al. (2001) characterized the **dopamine receptor D₅ null mutant** of mice in behavioral tests.

Based on pharmacology of adenosine A_{2A} receptor antagonists and results with **A_{2A} receptor knockout mice**, El Yacoubi et al. (2001) claimed that adenosine A_{2A} receptor antagonists are potential depressants.

MacQueen et al. (2001) investigated the performance of heterozygous **brain-derived neurotrophic factor knockout mice** on behavioral analogs of anxiety, nociception, and depression.

Conti et al. (2002) reported that cAMP-response-element-binding protein is essential for the upregulation of **brain-derived neurotrophic factor** transcription, but not the behavioral or endocrine responses to antidepressant drugs. cAMP-response-element-binding-protein-deficient mice were used to study the effects of desipramine and fluoxetine in behavioral, endocrine and molecular analyses.

Stork et al. (2000) studied postnatal development of a GABA deficit and disturbance of neural functions in mice lacking GAD65. Adult **GAD65(−/−) mice** showed a largely abnormal neural activity with frequent paroxysmal discharges and spontaneous seizures. They furthermore displayed increased anxiety-like behavior in a light/dark avoidance test and reduced inter-male aggression, as well as reduced forced-swimming-induced immobility indicative of an antidepressant-like behavior.

Svenningsson et al. (2002) studied the involvement of striatal and extrastriatal DARPP-32, a cAMP-regulated phosphoprotein, in biochemical and behavioral effects of fluoxetine using **DARPP-32 knockout mice**.

Calapai et al. (2001) used **interleukin-6 knockout mice** to characterize the antidepressant action of *Hypericum perforatum*.

Porsolt (2000) reviewed the utility of animal models of depression for transgenic research.

El Yacoubi et al. (2003) published the behavioral, neurochemical, and electrophysiological characterization of a genetic mouse model of depression.

Wei et al. (2004) generated mice overexpressing glucocorticoid receptor specifically in forebrain. These mice display a significant increase in anxiety-like and depression-like behaviors relative to wild type.

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E.7

Anti-Parkinsonism Activity

E.7.0.1

General Considerations

A fundamental lesion in Parkinson's disease is a marked deficiency in the dopaminergic innervation of the basal ganglia owing to degeneration of neurons in the substantia nigra. Enhancement of dopaminergic transmission restores motor function at least partially. The decrease in dopaminergic activity in the basal ganglia results in a relative excess of cholinergic influence. Therefore, dopaminergic agonists, such as levodopa a precursor of dopamine, and cholinergic (muscarinic) antagonists can be combined in the treatment of Parkinson's disease. Parkinson-like syndromes also occur after depletion of central stores by reserpine and after treatment with phenothiazines and other antipsychotic drugs blocking dopamine receptors. (Vernier 1964; Marsden et al. 1975; Duvoisin 1976; Hornykiewicz 1975; Miller and Hiley 1975). The pathology of Parkinson's disease is typified by the presence of cytoplasmic inclusions (Lewy bod-

ies). The formation of these proteinaceous inclusions involves the interaction of several proteins, including α -synuclein, synphilin, parkin and ubiquitin carboxyl-terminal hydrolases (Goldberg and Lansbury 2000; Shimohama et al. 2003; Le and Appel 2004; Meredith et al. 2004; Snyder and Wolozin 2004; von Bohlen und Halbach et al. 2004). Orr et al. (2002) gave a review on inflammatory aspects of Parkinson's disease and highlighted the cell-to-cell interactions and immune regulations critical for neuronal homeostasis and survival. Parkinson's disease and related synucleinopathies are considered as a new class of nervous system amyloidoses (Trojanowski and Lee 2002; Dev et al. 2004; Liu et al. 2005).

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E.7.1

In Vitro Methods

E.7.1.1

Culture of Substantia Nigra

PURPOSE AND RATIONALE

Although *in vivo* animal studies have been used to examine the effects of a number of Parkinson-inducing compounds, there is little information on reliable *in vitro* methodologies that can recapitulate the previously observed *in vivo* results. Cardozo (1993) and Smeyne and Smeyne (2002) described details of a method for generating mixed and chimeric neuron/glia cultures of postnatal substantia nigra (SN), independent of other monoaminergic nuclei in the ventral midbrain. Since many toxins do not affect regions of the midbrain except the SN, use of whole ventral midbrain from embryos can dilute any measurement of cell death. By specifically culturing ventrolateral midbrain containing the SN, one can more directly target the effects of dopaminergic toxins. In addition, this method can be used to test potential therapies for amelioration of Parkinson's disease.

PROCEDURE

Generation of SN Cultures

1. Postnatal day 2–5 C57Bl/6 mice and/or Swiss-Webster (SWR) mice (Harlan) are placed on ice for 2–3 min to achieve an appropriate plane of anesthesia.
2. Animals are quickly decapitated and \approx 8–10 brains from P2–P5 C57Bl/6 or SWR matings are removed and placed in a freshly prepared dissociation media (=DM containing 12.8 g sodium sulfate; 5.2 g potassium sulfate; 0.036 g calcium chloride · 2H₂O; 1.18 g magnesium chloride · 6H₂O; 1.8 g glucose anhydrous; 0.238 g HEPES in 1 l deionized water, adjusted to pH 7.4 with NaOH).
3. Brains are placed on their ventral surface and a slice of midbrain rostral to the cerebellum and caudal to the hippocampus is isolated. This removed brain slab is placed flat in DM and either the entire midbrain or the ventrolateral midbrain containing the SN is dissected and minced into small pieces.
4. The minced SN or midbrain is then incubated in papain and DNAase (Dissociation Kit, Worthington Biochemical, Freehold, N.J., USA, follow kit

instructions) for 30 min at 37°C. A second incubation with fresh papain solution (30 min, 37°C) is followed by three rinses in DM, and one rinse in plating media (PM).

5. Tissue is triturated in 5 ml PM and the cell suspension is added to 2 ml (1 ml of BSA stock and 1 ml ovalbumin stock).
6. The cell suspension is spun 1400 rev/min for 8 min and the pellet is then resuspended in 1.0 ml plating media containing 2% rat serum.
7. Once resuspended, cells are counted using trypan blue (0.4%) to determine cell viability.
8. Cells are adjusted to 1.2×10^6 cells/ml and plated at \approx 200,000 cells/cm² in Lab-Tek 4-well Permanox™ chamber slides that were previously coated with laminin (200 mg/ml, Collaborative Biomedical Products) and poly-D-lysine (200 mg/ml, Collaborative Biomedical Products) 1:1 (v:v) and rinsed once with deionized water. Cells are maintained in an incubator at 37°C, 5% CO₂, and fed 2–3 times per week with feeding media complete with 2% rat serum (RS) by exchanging approximately one-fifth of the media (100 μ l).

Chimeric Neuron/Glia Cultures

1. SNpc cells plated on pre-plated SNpc glia are produced using a variation of the previously described methods. SNpc glial feeder layers from P2–5 C57Bl/6 or SWR mice are produced using the above described method, but cells are plated at 20,000–50,000 cells/cm² and fed with plating media containing 2% rat serum and 8% fetal bovine serum to promote glial proliferation and neuronal death.
2. For astrocyte cultures, this is the final step.
3. To generate mixed neuron/glia cultures, 3–4 weeks after the initial SNpc cells are plated, the glial feeder layers are rinsed once with plating media without serum, and 0.5 ml of plating media with 2% rat serum (RS) are added.
4. SNpc cells from C57Bl/6 or SWR mice are isolated (as above) and plated at 250,000 cells/well on the previously generated glial feeding layers.
5. Twenty-four hours after plating of neurons onto the glial feeder layers, the cultures are fed with feeding media complete with 2% RS by an exchange of approximately one-fifth of the medium (100 μ l) and cytosine β -D-arabinofuranoside (Ara-C, 2 μ M) to prevent glial proliferation of the freshly plated cells. Thereafter, Ara-C (10 μ M, final concentration) is added at each feeding.

MPTP Treatment

- 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is added to cultures 7–10 days after the neurons are plated onto glia. The MPTP is prepared by dissolving MPTP in feeding media for a 1-mM stock, then diluting to a 1- μ M stock with feeding media complete with 2% rat serum and adding this directly to the cultures. The final concentration of the MPTP in media is 50 nM. Two feedings of MPTP, in 2 days, are necessary to achieve the desired toxic effect.
- Seven days after MPTP is added, the cultures are rinsed 3 \times with TBS, fixed in 4% buffered paraformaldehyde for 10 min, and rinsed 3 \times with TBS.

EVALUATION**Identification of SN Cells**

- To determine the number of SN cells, cultures are immunostained for expression of tyrosine hydroxylase. First, endogenous peroxidase activity is quenched by rinsing with 0.3% hydrogen peroxide in methanol in 1 \times TBS for 2 \times 15 min. Cells are permeabilized with 0.1% Triton X-100, 5% goat serum in TBS for 2 \times 15 min. Cultures are covered in 400 μ l of a polyclonal antibody directed against tyrosine hydroxylase (Eugene Tech International, Ramsey, N.J., USA or Pel-Freeze, Burlingame, Calif., USA: each 1:500) and incubated in this solution overnight at 4°C. The next day, cultures are rinsed three times with TBS followed by application of secondary antibody (goat α -rabbit) and amplification with Avidin–Biotin (ABC Elite Peroxidase kit, Vector Labs, Burlingame, Calif., USA). Final visualization of the immunopositive neurons is made using diaminobenzidine (DAB kit, Vector Labs) as a chromagen.
- All TH-positive cells having the previously described characteristics of SNpc neurons (TH-positive cytoplasm surrounding a pale unstained nucleus, Hamre et al. 1999) from each culture are counted at a magnification of 200 \times . Note: to control for variability between cultures, all MPTP treatments are performed on matched cultures present on one single slide. Once cells are counted in each well, the number of TH-positive cells in the MPTP-treated cultures from each genotype is compared to the adjacent non-treated culture. Since an identical number of cells generated from the same brains was plated on a single slide, these cultures are directly compared to determine the percentage cell loss following MPTP. The percentage cell loss data follow-

ing MPTP are then pooled from all C57Bl/6, SWR or mixed cultures.

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E.7.1.2**Inhibition of Apoptosis in Neuroblastoma SH-SY5Y Cells****PURPOSE AND RATIONALE**

A dopamine-derived neurotoxin, 1(*R*), 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline [*N*-methyl(*R*)salsolinol] was found to cause parkinsonism in rats and to deplete selectively dopamine neurons in the substantia nigra after infusion in the striatum. This isoquinoline occurs enantio-specifically in the nigra-striatum of human brains. The biosynthesis from dopamine is catalyzed by two enzymes, (*R*)salsolinol synthase and (*R*)salsolinol *N*-methyltransferase. The isoquinoline increases in the cerebrospinal fluid from parkinsonian patients, and the increase is ascribed to high activity of its synthesizing neutral (*R*)salsolinol *N*-methyltransferase, as shown by analyses in lymphocytes. The cell death caused by this neurotoxin in dopaminergic human neuroblastoma SH-SY5Y cells proved to be apoptotic. Apoptosis by this neurotoxin is mediated by intracellular sequential process, loss of mitochondrial membrane potential, activation of caspases and DNA fragmentation (Maruyama et al. 1997; Naoi et al. 1996, 1997, 2000, 2002).

Neuroprotection to halt progressive cell death of neurons has been proposed as a future therapy for neurodegenerative disorders. In these disorders, such as Parkinson's disease and Alzheimer's disease, apoptosis contributes to neuronal death in most cases (Tatton 2000). The well-regulated and relatively slow apoptotic process was proposed as a target of neuroprotection (Thompson 1995; Naoi and Maruyama 2001). Apoptosis is induced in neurons by various insults: oxidative stress, metabolic compromise, excitotoxicity, and neurotoxins. Apoptotic signaling is a multi-step pathway induced by opening a mitochondrial megachannel called permeability transition (PT) pore, followed by decline in membrane potential, $\Delta\Psi_m$, re-

lease of apoptosis-inducing factors, activation of caspases and fragmentation of nuclear DNA. Mitochondrial PT pore is regulated by Bcl-2 protein family, preventively by Bcl-2 and Bcl-xL and in a promotive way by BAX and BAD. Several papers describe the neuroprotective-antiapoptotic action of the anti-Parkinson drug rasagiline (Akao et al. 2002a, 2002b; Youdim et al. 2003; Maruyama et al. 2002, 2004b).

Maruyama et al. (2004a) described the neuroprotective function of *R*-(-)-1-(benzofuran-2-yl)-2-propylaminopentane, [*R*-(-)-BPAP], against apoptosis induced by *N*-methyl(*R*)salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells.

PROCEDURE

SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in an atmosphere of 95% air /5% CO₂.

Assessment of Apoptosis Induced by NM(*R*)Sal and the Protection by BPAP Derivatives

Apoptosis was quantitatively measured by fluorescence-augmented flow cytometry (FACS) with FACScan and CellQuest software (Becton Dickinson, San Jose, Calif., USA). Cells cultured in a 6-well poly-L-lysine-coated culture flask were incubated with or without 1 μM to 1 nM (-)-BPAP analogs at 37°C for 30 min, and then for 24 h with 250 μM NM(*R*)Sal in Cosmedium-001 culture medium supplemented with fetal calf serum. The cells were treated with trypsin, gathered, washed with the culture medium and twice with phosphate-buffered saline (PBS). The cells were incubated with 100 nM test drugs solution in an ice-bath for 30 min, washed and suspended in PBS, then subjected to FACS analysis.

Measurement of Changes in $\Delta\Psi_m$

Decline in $\Delta\Psi_m$ induced by NM(*R*)Sal was quantified by measuring the reduction of Rhodamine 123 fluorescence pre-loaded in the cells (Patarino et al. 1996), as reported by Akao et al. (2002b). To examine the effects of (-)-BPAP analogs, the cells cultured in 6-well poly-L-lysine-coated tissue culture flasks were stained with 5 μM Rhodamine 123 in DMEM for 30 min at 37°C. After being washed twice with PBS, the cells were suspended in DMEM, incubated with 1 μM to 1 nM BPAP derivatives for 30 min, then with 250 μM NM(*R*)Sal for 1 h. After being washed and gathered by treatment with trypsin, the cells were suspended in PBS and the

fluorescence at 535 nm was measured with excitation at 505 nm in a Shimadzu spectrofluorophotometer, RF-5000 (Kyoto, Japan).

Measurement of bcl-2 mRNA Level in the Cells Treated with BPAP Derivatives

SH-SY5Y cells were cultured in the presence of various concentrations (100 nM to 10 pM) of (-)-BPAP analogs for 24 h, and mRNA levels of bcl-2 were quantitatively assessed by RT-PCR (Akao et al. 2002a, 2002b). The cells were gathered and washed with PBS, and the total RNA was extracted by the phenol/guanidinium thiocyanate method. cDNA was generated by reverse transcription of 2 μg of the total RNA, and the cDNA fragments were amplified using the PCR primers. The linearity of the relationship of the amount of PCR product to the time of PCR amplification was confirmed under the conditions used in this study. PCR products were analyzed by electrophoresis on 3% agarose gels, and α -actin was used as an internal standard. The amounts of mRNA were quantified using NIH imaging software (version 1.62, developed at the U.S. National Institute for Health).

Measurement of Bcl-2 Levels in the Cells Treated with (-)-BPAP Derivatives

SH-SY5Y cells treated with 1 μM to 1 pM (-)-BPAP analogs for 24 h, and the cells were gathered, washed with PBS and suspended in RIPA buffer (10 mM Tris-HCl buffer, pH 7.5, containing 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 150 mM NaCl and 1 mM EDTA 2Na). The lysed protein (5 μg) was separated by SDS-PAGE using a 10%–20% gradient polyacrylamide gel (Bio-Rad, Hercules, Calif., USA) and electroblotted onto PVDF membranes (Du Pont, Boston, Mass., USA). After blockage with 5% nonfat milk in PBS containing 0.1% Tween 20, the membrane was incubated overnight at 4°C with anti-human Bcl-2 (100) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), or anti- β -actin antibody as control (Sigma, St. Louis, Mo., USA). The membranes were incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, Wis., USA) at room temperature. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, Mass., USA), and quantified by computer-assisted image analysis with the NIH imaging software.

EVALUATION

Experiments were repeated four to eight times, and the results were expressed as the mean and SD. Differences were statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's *F*-test. A *p* value less than 0.05 was considered to be statistically significant.

MODIFICATIONS OF THE METHOD

Maruyama et al. (2000) found that dopaminergic neurotoxins, 6,7-dihydroxy-1-(3',4'-dihydroxybenzyl)-isoquinolines, cause different cell death in SH-SY5Y cells: apoptosis was induced by oxidized papaverolines and necrosis by reduced tetrahydropapaverolines.

Maruyama et al. (2003) described the anti-apoptotic action of an anti-Alzheimer drug, TV3326 [(N-propargyl)-(3R)-aminoindan-5-yl)-ethyl methyl carbamate], a novel cholinesterase-monoamine oxidase inhibitor.

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E.7.2**In Vivo Methods****E.7.2.1****Tremorine and Oxotremorine Antagonism****PURPOSE AND RATIONALE**

The muscarinic agonists tremorine and oxotremorine induce parkinsonism-like signs such as tremor, ataxia, spasticity, salivation, lacrimation and hypothermia. These signs are antagonized by anticholinergic drugs.

PROCEDURE

Groups of six to ten male NMRI mice weighing 18–22 g are used. They are dosed orally with the test compound or the standard (5 mg/kg benzatropine mesilate) 1 h prior to the administration of 0.5 mg/kg oxotremorine s.c. Rectal temperature is measured before administration of the compound (basal value) and 1, 2 and 3 h after oxotremorine injection. Tremor is scored after oxotremorine dosage in 10-s observation periods every 15 min for 1 h.

| Tremor | Score |
|--------|-------|
| absent | 0 |
| slight | 1 |
| medium | 2 |
| severe | 3 |

Salivation and lacrimation are scored 15 and 30 min after oxotremorine injection.

| | |
|--------|---|
| absent | 0 |
| slight | 1 |
| medium | 2 |
| severe | 3 |

EVALUATION

Hypothermia

The differences of body temperature after 1, 2 and 3 h versus basal values are summarized for each animal in the control group and the test groups. The average values are compared statistically.

Tremor

The scores for all animals in each group at the three observation periods are summarized. The numbers in the treated groups are expressed as percentage of the number of the control group.

Salivation and Lacrimation

The scores for both symptoms for all animals in each group are summarized at the two observation periods. The numbers in the treated groups are expressed as a percentage of the number of the control group.

CRITICAL ASSESSMENT OF THE METHOD

The oxotremorine antagonism has been proven to be a reliable method for testing central anticholinergic activity. The overt isomorphism between the animal model and the symptoms of Parkinson's disease recommend this test for screening of anti-Parkinson drugs (Cho et al. 1962; Vernier 1964; Everett 1964; Turner 1965; Bebbington et al. 1996; Frances et al. 1980; Ringdahl and Jenden 1983). However, the model measures only central anticholinergic activity (Duvoisin 1976).

MODIFICATIONS OF THE METHOD

Matthews and Chiou (1979) developed a method for quantifying resting tremors in a rat model of limb dyskinesias. The model involved permanent cannulation of the caudate nucleus for the introduction of carbachol. Tremors were quantified with a small

transducer and an electronic data collecting system. The system allows the construction of dose-response curves for tremor inhibition by potential antiparkinsonism drugs.

Johnson et al. (1986) developed a procedure for quantifying whole-body tremors in mice. Displacement of a free floating platform by animal movement created a change in resistance across a strain gauge. Administration of oxotremorine, 2.5 mg/kg, i.p, produced numerous high-frequency, high-intensity peaks within 5 min.

Clement and Dyck (1989) constructed and tested a tremor monitor that quantitates soman- and oxotremorine-induced tremors. The device consisted of a force transducer, from which a plastic beaker was suspended containing a mouse. The signal from the force transducer was fed into a tremor monitor and quantitated using the Applecounter from Columbus Instruments.

Coward et al. (1977) recommended *N*-carbamoyl-2-(2,6-dichlorophenyl)acetamide hydrochloride (LON-954), a tremorigenic agent, as an alternative to oxotremorine for the detection of anti-Parkinson drugs.

Rats treated with 3-acetylpyridine show a selective degeneration of neurons in the inferior olive nucleus and the olivo-cerebellar tract with characteristic motor inco-ordination and ataxia (Denk et al. 1968; Watanabe et al. 1997; Kinoshita et al. 1998). Similar motor dysfunction is seen in patients with olivoponto-cerebellar atrophy. To measure the effect of 3-acetylpyridine and the ameliorating effect of TRH agonists in rats the maximal height of vertical jump after stimulation by a foot shock was measured.

Stanford and Fowler (1997) used a special technique for measuring forelimb tremor in rats induced by low doses of physostigmine. The rats pressed a force-sensing operandum while a computer measured force output and performed Fourier analyses on resulting force-time waveforms.

Several studies indicate that the exposure to the pesticide **rotenone** causes highly selective dopaminergic degeneration and α -synuclein aggregation in rats (Bertarbet et al. 2000; Sherer et al. 2003a, 2003b).

Tremulous jaw movements in rats induced by tacrine (Mayorga et al. 1997; Cousins et al. 1999) are considered to reflect symptoms in Parkinson's disease. They are suppressed by dopamine agonists (Salamone et al. 2005).

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E.7.2.2

MPTP Model of Parkinson's Disease

PURPOSE AND RATIONALE

MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has been shown to cause symptoms of Parkinson's disease in exposed individuals. The first observation was made in a relatively young, drug-abusing population. This initiated many clinical and experimental studies (Chiba et al. 1984; Heikkila et al. 1984; Kindt et al. 1988; Review by Special 2002). When administered to primates this compound causes a partial destruction of basal ganglia and a syndrome that resembles Parkinson's disease.

PROCEDURE

Burns et al. (1983) treated eight adult rhesus monkeys weighing 5–8 kg over a period of 5–8 days with cumulative intravenous doses of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (N-MPTP) up to 10–18 mg/kg. These animals showed a parkinsonism-like disorder (akinesia, rigidity, postural tremor, flexed posture, eyelid closure, drooling) which was reversed by the administration of L-dopa. The pathological and biochemical changes produced by N-MPTP are similar to the well established changes in patients with Parkinsonism.

The N-MPTP intoxication was applied using marmosets by Nomoto et al. (1985, 1988), Temlett et al. (1989) and by Keabian et al. (1992) to evaluate potential antiparkinson drugs.

EVALUATION

The severity of parkinsonian symptoms is rated by trained observers using a scale of 0 (normal) to 17 (maximum severity) that assesses movement (0: normal; 1: reduced; 2: sleepy), checking movements (0: present; 1: reduced; 2: absent), attention and blinking (0: normal; 1: abnormal), posture (0: normal; 1: abnormal trunk; 2: abnormal trunk and tail; 3: abnormal trunk, tail, and limbs; 4: flexed posture), balance and coordination (0: normal; 1: impaired; 2: unstable;

4: falls), reactions (0: normal; 1: reduced; 2: slow; 3: absent) and vocalizations (0: normal; 1: reduced; 2: absent).

MODIFICATIONS OF THE METHOD

Close and Elliott (1991) studied the behavioral effects of anti-Parkinsonian drugs in normal and MPTP-treated marmosets following central microinfusions.

Kebabian et al. (1992) tested a selective D₁ receptor agonist with antiparkinsonian activity in MTPT-treated marmosets.

Domino and Sheng (1993) studied the relative potency of some dopamine agonists with varying selectivities for D₁ and D₂ receptors in MPTP-induced hemi-parkinsonian monkeys.

Gnanalingham et al. (1995) used MTPT-treated marmosets and found differential effects with D₁ dopamine antagonists as compared with the effects in rats with unilateral 6-OGDA-induced lesions, where 6-OGDA is 6-hydroxydopamine.

Doudet et al. (1993) used intravenous administration of ¹⁵O-labeled water and 6-(¹⁸F)-L-fluorodopa to assess abnormal striatal activity in monkeys after long-term recovery of unilateral lesions of the dopaminergic nigro-striatal system induced by MPTP. Positron emission tomography (PET) data were examined in relation to behavioral and biological parameters, such as cerebral blood flow.

Belluzzi et al. (1994) induced a hemiparkinsonian syndrome in *Macaca nemestrina* monkeys by unilateral infusion of MPTP into the right coronary artery.

Raz et al. (2000) recorded hand tremor and simultaneous activity of several neurons in the external and internal segments of the globus pallidus in **vervet monkeys** before and after treatment with MPTP.

Rollema et al. (1989) compared the effects of intracerebrally administered MPP⁺ (1-methyl-4-phenylpyridinium) in three species (**mouse**, **rat** and **monkey**) by microdialysis determinations of dopamine and metabolites in the striatum.

Asin et al. (1997) tested a selective D₁ receptor agonist in **rats** previously given unilateral 6-hydroxydopamine injections and in **macaques** previously given unilateral, intracarotid infusions of MPTP.

Lange (1989, 1990) described circling behavior in old **rats** after unilateral intranigral injection of MPTP.

Fuxe et al. (1992) studied the protection against MPTP-induced degeneration of the nigrostriatal dopamine neurons in the **black mouse**.

There are marked species differences in susceptibility to the neurotoxic effects of MTPT (Giovanni et al. 1994; Schober 2005).

Grünblatt et al. (2001) described gene expression analysis of mice in the MPTP model of Parkinson's disease using cDNA microarray.

Mandel et al. (2003) reviewed the use of cDNA microarrays to assess Parkinson's disease models and the effects of neuroprotective drugs.

Hamre et al. (1999) performed a quantitative analysis in seven strains of **mice** and found that differential strain susceptibility following MPTP administration acts in an autosomal fashion.

Sedelis et al. (2001) described behavioral phenotyping of the MPTP mouse model on Parkinson's disease.

Muramatu et al. (2003) published an immunocytochemical study on cerebral alterations in an MPTP mouse model of Parkinson's disease.

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E.7.2.3

Reserpine Antagonism

PURPOSE AND RATIONALE

Reserpine induces depletion of central catecholamine stores. The sedative effect can be observed in mice shortly after injection, followed by signs of eyelid ptosis, hypokinesia, rigidity, catatonia, and immobility. These phenomena can be antagonized by dopamine agonists.

PROCEDURE

Male NMRI mice of either sex weighing 20–25 g are used. They are injected intraperitoneally with 5 mg/kg reserpine and tested 24 h later. The test compounds are injected 30 min prior to observation. The animals are placed singly onto the floor of a Perspex container (30 × 26 cm, 20 cm high), situated on a Panlab proximity sensor unit. Horizontal movements are recorded for 10 min. Moreover, rearings and grooming episodes are recorded by an experienced observer.

EVALUATION

Locomotor activity and grooming scores of drug-treated animals are compared with controls treated with reserpine and vehicle only by analysis of variance.

MODIFICATIONS OF THE METHOD

Rats treated with reserpine develop spontaneous orofacial dyskinesia that has features similar to tardive dyskinesia in humans (Abbot et al. 1991; Nisewander et al. 1994). The incidence of tongue protrusions was recorded to quantify the occurrence of oral dyskinesia.

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E.7.2.4

Circling Behavior in Nigrostriatal Lesioned Rats

PURPOSE AND RATIONALE

Unilateral lesion of the dopaminergic nigrostriatal pathway in the rat by the neurotoxin 6-hydroxydopamine (6-OHDA) induces hypersensitivity of the postsynaptic dopaminergic receptors in the striatum of the lesioned side (Ungerstedt 1971). The rats rotate in a direction towards the lesioned side (ipsilateral) when an indirect acting compound such as amphetamine is administered, but to the opposite direction (contralateral) when a directly acting dopamine agonist, e. g., apomorphine, or the dopamine precursor L-dopa is given. Therefore, this test can be used for the study of central dopamine function and the evaluation of dopamine antagonists and agonists, particularly the activity of novel antiparkinsonian drugs.

PROCEDURE

Male Wistar rats weighing 200–250 g at the time of surgery are used. They are housed individually in a controlled environment with free access to food and water.

The animals are anesthetized with sodium pentobarbital. The head is placed in a stereotaxic device (DKI 900) and positioned according to the atlas of König and Klippel (1963). After a sagittal cut is made in the skin of the skull, a 2-mm-wide hole is drilled with an electrical trepan drill. Care is taken not to lesion the meninges. A 30-gauge stainless-steel cannula connected to a Hamilton syringe is aimed at the anterior zona compacta of the substantia nigra (coordinates anterior 4.1, lateral 1.0 and dorso-ventral –2.5 from instrument zero). A total of 8 µg of 6-OHDA in 4 µl of saline is injected at a rate of 1 µl/4 min. After the intracranial injection the wound is closed. The animal is allowed several weeks for recovery and for development of the lesion.

Specially constructed opaque plastic spheres attached to solid-state programming equipment serve as test chambers. The number of full turns, either ipsilateral or contralateral to the lesion, is recorded on an

automatic printout counter every 15 min for 1- or 2-h test sessions.

To determine the control values for ipsilateral turning, each subject is administered 2.5 mg/kg of d-amphetamine and immediately placed in the circling chamber for 2 h. Control values for contralateral circling are determined by injecting apomorphine at 1 mg/kg s.c. and recording the rat's circling for 1 h.

Test compounds are given i.p. or s.c. and the animals placed into the circling chambers. Circling is recorded over a 1-h period.

EVALUATION

Percent change of drug turns from control turns is recorded. Using various doses ED₅₀ values can be calculated.

MODIFICATIONS OF THE METHOD

Etemadzadeh et al. (1989) described a computerized rotometer apparatus for recording circling behavior. The digital pulses derived from the infrared photocell detector induced by the animal rotations were fed directly to a 20-megabyte microcomputer for online recording and were processed further to the Digital Equipment Corporation's VAX computer with the SAS software system for statistical and graphical analysis.

Hudson et al. (1993) described a 16-channel automated rotometer system for reliable measurement of turning behavior in 6-hydroxydopamine-lesioned and transplanted rats. The system is preferable to more tedious methods such as videotaping and subsequent manual analysis or various other mechanical systems.

A rotometer differentiating between clockwise and counter-clockwise rotations with computerized evaluation is available from Technical and Scientific Equipment (Bad Homburg, Germany; Schwarz et al. 1978).

Carey (1989) tested stimulant drugs as conditioned and unconditioned stimuli in a classical conditioning paradigm using drug-induced rotational behavior in rats with unilateral lesions of dopamine neurons.

The production of asymmetry and circling behavior following unilateral, intrastriatal administration of neuroleptics was discussed by Costall et al. (1983).

Rotational behavior produced by intranigral injections of bovine and human β-casomorphins in rats was described by Herrera-Marschitz et al. (1989).

Perese et al. (1989) created a hemiparkinsonian model in rats in which there is 6-OHDA-induced destruction of the dopaminergic nigrostriatal pathway but sparing of the dopaminergic mesolimbic pathway.

Sauer and Oertel (1994) published a retrograde tracing and immunocytochemical study on the pro-

gressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine in the rat.

Garrett and Holtzman (1996) compared the effects of apomorphine, d-amphetamine, cocaine and caffeine on locomotor activity and rotational behavior in rats with unilateral 6-OHDA-induced lesions of the nigrostriatal tract.

McElroy and Ward (1995) reported that the high-affinity and selective dopamine D₃ receptor ligand, 7-OH-DPAT, caused 6-OHDA-lesioned rats to rotate in a direction contralateral to the lesioned side similarly to the direct-acting dopamine agonist apomorphine.

Haque et al. (1996) directly infused the neurotrophins NT3 and NT4/5 intraparenchymally in close proximity to transplanted nigral tissue placed in the dopamine-depleted striatum of 6-hydroxydopamine-lesioned rats.

A survey on the unilateral 6-hydroxydopamine lesion model in behavioral brain research was prepared by Schwarting and Huston (1996).

DSP-4 [*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine] is highly toxic to noradrenergic neurons (Finnegan et al. 1999). Srinivasan and Schmidt (2003, 2004) showed that parkinsonian symptoms in 6-hydroxydopamine-induced partial degeneration of substantia nigra in rats are potentiated after depletion of nucleus coeruleus noradrenaline by intraperitoneal treatment with DPS-4.

Harro et al. (2003) studied the effect of denervation of the locus coeruleus projections by DSP-4 treatment on [³H]-raclopride binding to dopamine D₂ receptors and D₂ receptor–G protein interaction in rat striatum.

Löscher et al. (1996) described a mutant rat strain (*CI*) with abnormal circling behavior reminiscent of 6-hydroxydopamine-lesioned rats.

Mele et al. (1997) studied alterations in striatal dopamine overflow by *in vivo* microdialysis during rotational behavior of rats induced by amphetamine, phencyclidine and MK 801.

Smith et al. (1996) reported contralateral turning in chronically cannulated rats after stimulation of glutamate receptors by unilateral intrastriatal injections of glutamate receptor agonists, such as kainate or AMPA.

Spooren et al. (2000) tested the effects of a prototypical mGlu₅ receptor antagonist on rotarod locomotor activity and rotational responses in unilateral 6-OHDA-lesioned rats using an accelerated rotarod and automated rotameter cylinders (TSE Systems, Bad Homburg, Germany).

Using the same equipment, Breyse et al. (2002) reported that chronic but not acute treatment with

a metabotropic glutamate 5 receptor antagonist reverses the akinetic deficits in rats with 6-hydroxydopamine lesions.

Using automated rotameter bowls (TSE Systems, Bad Homburg, Germany), Meissner et al. (2002) found that deep brain stimulation of subthalamic neurons induces contralateral circling in freely moving 6-hydroxydopamine-lesioned rats.

With this equipment, Lebsanft et al. (2005) found, that 3,4-methylenedioxymethylamphetamine counteracts akinesia enantioselectively in rat rotational behavior.

O'Neill et al. (2004) described neurotrophic actions of an AMPA receptor potentiator, which may increase brain-derived neurotrophic factor expression, in rodent models of Parkinson's disease.

Inhibition of sinistrotorsion induced in **guinea pigs** by injection of physostigmine into the right carotid artery was proposed as a method of screening central anticholinergic activity by De Jonge and Funcke (1962).

Behavioral quantification of striatal dopaminergic supersensitivity after bilateral 6-hydroxydopamine lesions in the **mouse** was reported by Mandel et al. (1992).

Worms et al. (1986) and Poncet et al. (1993) studied turning behavior in mice induced by intrastriatal injection of neuropeptides.

Fitzgerald et al. (1992) recommended the **chakragati mouse (ckr)**, a transgenic insertional mutant which displays lateral circling, locomotor hyperactivity and hyperreactivity, as a model to study aspects of neuropsychiatric disorders associated with dopaminergic abnormalities.

Emonds-Alt et al. (1995) injected 0.3 pg senktide into the striatum of **gerbils** inducing contralateral rotations which were antagonized by intraperitoneal or oral administration of a tachykinin NK₃ receptor antagonist.

Vernier and Unna (1963) tested drugs against the tremor induced by stereotactically oriented electric lesions in the region of the subthalamus or the mesencephalic reticular formation in **monkeys**.

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E.7.2.5**Elevated Body Swing Test****PURPOSE AND RATIONALE**

Borlongan and Sanberg (1995); Borlongan et al. (1995) proposed the elevated body swing test as a measure of asymmetrical motor behavior of hemiparkinsonian animals in a drug-free state.

PROCEDURE

Male, 8-week-old Sprague Dawley rats are anesthetized with sodium pentobarbital (60 mg/kg i.p.) and mounted in a Kopf stereotaxic frame. They are lesioned by injection of 8 µg 6-hydroxydopamine in 4 µl saline containing 0.02% ascorbic acid in the left substantia nigra (AP – 5.0, ML+1.5, DV – 8.0). The solution is injected over a 4-min period and the needle left in place for an additional 5 min before retraction.

Seven days after the lesion, behavioral testing is performed. The animal is allowed to habituate in a Plexiglas box and attain a neutral position having all four paws on ground. The rat is held about 2.5 cm from the base of its tail and elevated 2.5 cm above the surface on which it has been resting. A swing is recorded whenever the animal moves its head out of the vertical

axis to either side. Before attempting another swing, the animal must return to the vertical position for the next swing to be counted. Swings are counted for 60 s over four consecutive 15-s segments. The total number of swings made to each side is divided by the overall total number of swings made to both sides to get percentages of left and right swings. The criterion of biased swing is set at 70% or higher. At 30 and 45 s, 6-OHDA-lesioned rats exhibit right-biased swings of 70% or higher compared to normal rats.

EVALUATION

A two-way ANOVA is used to analyze swing behavior data across the 15-s segments.

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E.7.2.6**Skilled Paw Reaching in Rats****PURPOSE AND RATIONALE**

Montoya et al. (1990, 1991), Abrous et al. (1993), Abrous and Dunnett (1994), Nikkhah et al. (1993), and Barnéoud et al. (1996) used the skilled paw reaching test as a model of Parkinson's disease in the rat. The term "staircase test" mentioned in this context has nothing to do with the staircase test described by Thiebot et al. (1973) for evaluating anxiolytic activity in rats (see E.2.4.6).

Unilateral injection of 6-OHDA into the medial forebrain bundle results in an impairment of paw reaching on both sides which can be ameliorated by drug treatment or transplantation of a nigral cell suspension.

The apparatus has been developed after earlier studies by Whishaw et al. (1986) who investigated the contributions of motor cortex, nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat.

PROCEDURE**Apparatus**

The apparatus consists of a clear Perspex chamber with a hinged lid. A narrower compartment with a central platform running along its length, creating a trough

on either side, is connected to the chamber. The narrowness of the side compartment prevents rats from turning around, so they can use only their left paw for reaching into the left trough and their right paw for reaching into the right trough. A removable double staircase is inserted into the end of the box, sliding into the troughs on either side of the central platform. Each of the steps of the staircase contains a small well, and two 45-mg saccharin-flavored pellets are placed in each well.

Learning Procedure

The week before the start of the training period, the rats are deprived of food and their body weight is stabilized at 85% of the weight of non-deprived rats. At the same time, they are gently manipulated and familiarized with the appetitive saccharin-flavored pellets.

The animals then begin to learn the paw reaching task. For 4 weeks they are placed in the test boxes once per day for 10–15 min. The number of pellets eaten during the test period indicates the rat's success in grasping and retrieving the pellets; the number of steps from which pellets have been removed provides an index of the attempts to reach the food and how far the rat can reach; the number of missed pellets remaining at the end of the test on the floor of the side compartment indicates a lack of sensorimotor coordination in grasping and retrieving the pellets. In addition to these three parameters, it is noted which forepaw the rat used for the first movement to reach the pellet on each test day. A first choice score of +1 corresponds to the paw contralateral to the lesion, a score of -1, to the paw ipsilateral to the lesion. Because rodents exhibit a "pawedness," it must be noted whether there is a preference for one paw.

Lesions

The mesotelencephalic system is lesioned by a stereotaxic unilateral injection of 6-OHDA into the medial forebrain bundle under equithesin anesthesia. 6-OHDA is injected in a volume of 1.5 μ l and at a concentration of 4 μ g/ μ l of 0.9% saline and 0.01% ascorbic acid twice over 3 min via a 30-gauge stainless steel cannula at the stereotaxic coordinates: $L = 1.6$ mm, $AP = 0$ mm, $V = -7.6$ mm and $L = 1.6$ mm, $AP = -1$ mm, $V = -8$ mm. The coordinates AP and L are estimated relative to the bregma, and V is measured from the level of the dura, with the incisor bar set 5 mm above the interaural line. Following each injection, the cannula is left in place for an additional 4 min to allow the diffusion of the neurotoxin away from the injection site. The sham-operated group receives sham le-

sions by identical injection of ascorbate-saline solution alone.

Drug Treatment

The animals are injected i.p. with the test drug or saline 30 min before the unilateral 6-OHDA lesion and 24 h thereafter.

EVALUATION

Test sessions are performed 4, 5, 7, and 8 weeks after 6-OHDA lesion. The parameters success, attempts and sensorimotor coordination are subjected to a two-way ANOVA with group as the independent measure and weeks as the dependent measure.

MODIFICATIONS OF THE METHOD

Fricker et al. (1996) investigated the effect of unilateral ibotenic acid lesions in the dorsal striatum, placed at anterior, posterior, medial, or lateral loci, in the staircase test of skilled forelimb use.

Nakao et al. (1996) studied paw reaching ability in rats with unilateral quinolinic acid lesions of the striatum as an animal model for Huntington's disease.

Barnéoud et al. (1996) evaluated the neuroprotective effects of riluzole using impaired skilled forelimb use, circling behavior, and altered dopaminergic metabolism of the mesotelencephalic system in unilaterally 6-OHDA-lesioned rats.

Fricker et al. (1997) studied the correlation between positron emission tomography, using ligands to the D_1 and D_2 receptors, and reaching behavior in rats with ibotenic acid lesions and embryonic striatal grafts.

Grabowski et al. (1993), Marston et al. (1995), and Sharkey et al. (1996) tested drug effects on skilled motor deficits produced by middle cerebral artery occlusion in rats using the paw reaching test.

Meyer et al. (1997) described a revolving food pellet test for measuring sensorimotor performance in rats.

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PROCEDURE

6-OHDA Lesion Surgery

Female Sprague Dawley rats receive two stereotaxic injections of 6-OHDA (3.6 µg/µl in 0.2 µg/ml ascorbate-saline) into the right ascending mesostriatal dopamine pathway using a 10-µl Hamilton syringe at the following coordinates (in mm, with reference to bregma and dura):

1. 2.5 µl at AP -4.4, L 1.2, V 7.8, tooth bar -2.4;
2. 3.0 µl at AP -4.0, L 0.8, V 8.0, tooth bar +3.4 at an injection rate of 1 µl/min.

The cannula is left in place for an additional 5 min before slowly retracted.

Experimental Setup for Stepping Test

The tests monitoring initiation time, stepping time and step length are performed using a wooden ramp with a length of 1 m connected to the rat's home cage. A smooth-surfaced table is used for measuring adjusted steps.

During the first 3 days the rats are handled by the experimenter to familiarize them with the experimenter's grip. During the subsequent 1–2 days the rats are trained to run spontaneously up the ramp to the home cage.

The stepping test comprises two parts: first, the time to initiation of a movement of each forelimb, the step length, and the time required for the rat to cover a set distance along the ramp with each forelimb; and second the initiation of adjusting steps by each forelimb when the animal was moved sideways along the bench surface. Each test consists of two tests per day for three consecutive days and the mean of six subtests is calculated.

Initiation Time, Stepping Time, and Step Length

The rat is held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. The other hand fixes the forelimb not to be monitored. Time is measured until the rat initiates movement with the forelimb not fixed by the experimenter, using 180 s as break-off point. Stepping time is measured from initiation of movement until the rat reaches the home cage. Step length is calculated by dividing the length of the ramp by the number of steps required for the rat to run up the ramp. The sequence of testing is right paw testing followed by left paw testing, repeated twice.

Adjusting Steps

The rat is held in the same position as described above with one paw touching the table, and is then moved

E.7.2.7

Stepping Test in Rats

PURPOSE AND RATIONALE

Schallert et al. (1992), Olsson et al. (1995) and Rosenblad et al. (1997) introduced the stepping test as a clinically relevant unilateral model of Parkinsonian akinesia. The 6-OHDA lesion induced marked and long-lasting impairments in the initiation of stepping movements with the contralateral paw which can be ameliorated by the systemic application of drugs.

slowly sideways (5 s for 0.9 m) by the experimenter, first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand directions of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated twice each day.

The paw contralateral to the lesion is passively dragging when the rat is moved in the forehand direction, while the ipsilateral paw performs frequent stepping movements.

Drug Application

Stepping tests are repeated to determine the baseline weekly after the 6-OHDA lesion. The drug tests are administered for 1 day only. Various drugs can be evaluated in weekly intervals.

EVALUATION

Results are expressed as means \pm SEM. For statistical evaluation, the data are subjected to one-factor analysis of variance (ANOVA) and Fisher post hoc test.

MODIFICATIONS OF THE METHOD

Schallert et al. (2000), Picconi et al. (2003, 2004), and Centonze et al. (2005) described the limb-use asymmetry test to measure motor performance in rats with 6-OHDA-induced lesions. Forelimb use during exploratory activity was analyzed in a transparent cylinder (20 cm diameter and 30 cm height) for 3–10 min depending on the degree of movement maintained during the trial. The number of supporting wall contacts executed independently with the right of the left forelimb was counted. The percentage of wall contacts executed by the impaired forelimb (contralateral to the lesion) was then subtracted from the percentage of contacts with the non-impaired forelimb to obtain a limb-use asymmetry score.

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E.7.2.8

Transgenic Animal Models of Parkinson's Disease

PURPOSE AND RATIONALE

New insight in the pathobiology of Parkinson's disease has led to the development of transgenic animal models (Gonzalez-de Aguilar et al. 2003; Kirik and Björklund 2003; Levine et al. 2004).

The most prominent models are related to α -synuclein. The first transgenic mice that express human α -synuclein were generated by Masliah et al. (2000). These mice displayed a progressive accumulation of α -synuclein and ubiquitin-immunoreactive inclusions in neurons of the neocortex, hippocampus, and substantia nigra. These alterations were associated with a loss of dopaminergic terminals in the basal ganglia and with motor impairments. Since then, several studies on transgenic α -synuclein mice have been performed (van der Putten et al. 2000; Masliah et al. 2001; Giasson et al. 2002; Kirik et al. 2002; Gispert et al. 2003; Hashimoto et al. 2003; Fernagut and Chesselet 2004; Martin-Clemente et al. 2004; Cabin et al. 2005; Frasier et al. 2005; Poon et al. 2005). Masliah and Hashimoto (2002) discussed the possible role of β -synuclein for development of new treatments for Parkinson's disease using transgenic animal models.

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a neuron-specific ubiquitin recycling enzyme, which may be implicated in the pathogenesis of Parkinson's disease (Leroy et al. 1998). Lindsten et al. (2003) developed a model for *in vivo* analysis of the ubiquitin/proteasome system by generating mouse strains transgenic for a green fluorescence protein reporter carrying a constitutively active degradation signal.

Loss-of-function mutations in *parkin* are the major cause of early-onset familial Parkinson's disease. To investigate the pathogenic mechanism by which loss

of parkin function causes Parkinson's disease, Goldberg et al. (2003) generated a mouse model bearing a germline disruption in parkin. In addition to PARK1 (representing the α -synuclein gene) and PARK2 (representing the UCH-L1 gene), other monogenic forms of Parkinson's disease have been described (Lansbury and Brice 2002; von Bohlen und Halbach et al. 2004).

The *weaver mutant mouse* has been proposed as another model of Parkinson's disease (Bankiewicz et al. 1993; Bandmann et al. 1996; Cheng et al. 1997; Ebadi et al. 2005).

MODIFICATIONS OF THE METHOD

Lo Bianco et al. (2002) studied α -synucleinopathy and selective dopaminergic neuron loss in a *rat* lentiviral-based model of Parkinson's disease.

Kirik et al. (2003) described nigrostriatal α -synucleinopathy induced by viral vector-mediated overexpression of human α -synuclein using a primate (adult *marmosets*) model of Parkinson's disease.

Transgenic *Drosophila* models expressing human α -synuclein were described by Feany and Bender (2000), Pendleton et al. (2002), and Scherzer et al. (2003).

Brown et al. (2002) published multiplex three-dimensional brain gene expression mapping in a mouse model of Parkinson's disease.

Son et al. (2003) described cloning and expression analysis of the Parkinson's disease gene, *UCH-L1*, and its promoter in *zebrafish*.

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- In spite of almost 20 years of experimental experience, clinical trials with cell transplantation for Parkinson's disease have had disappointing results (Drucker-Colin and Verdugo-Diaz 2004; Linazaroso 2004; Roitberg et al. 2004).

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E.7.2.9

Cell Transplantations into Lesioned Animals

PURPOSE AND RATIONALE

Human embryonic stem cells may potentially serve as a renewable source of cells for transplantation. In Parkinson's disease, embryonic stem-cell-derived dopaminergic neurons may replace the degenerated neurons in the brain. To substantiate this goal, numerous animal experiments were performed. Most authors used rats rendered hemi-Parkinsonian by injection of 6-OHDA into the substantia nigra as recipients (Zawada et al. 1998; Mendez et al. 2000; Sawamoto et al. 2001; Björklund et al. 2002; Hao et al. 2002; Kim et al. 2002; Ben-Hur et al. 2004; Burnstein et al. 2004; Jollivet et al. 2004; Levy et al. 2004; Yoshizaki et al. 2004; Rafuse et al. 2005; Richardson et al. 2005).

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E.7.2.10

Transfer of Glial Cell Line-Derived Neurotrophic Factor (GDNF)

PURPOSE AND RATIONALE

Recombinant viral vectors derived from adenovirus, adeno-associated virus or lentivirus have been developed into highly effective vehicles for gene transfer to the adult central nervous system. These vectors have been shown to be effective for long-term delivery of glial cell-line derived neurotrophic factor (GDNF) in the nigrostriatal system. GDNF is a strong candidate agent in the neuroprotective treatment of Parkinson's disease. The injection of adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protected nigral neurons in the 6-OHDA-induced degeneration model of Parkinson's disease in rats (Yoshimoto et al. 1995; Bilanz-Bleuel et al. 1997; Lapchak et al. 1997; Mandel et al. 1997; Björklund et al. 2000; Chen et al. 2003; Wang et al. 2002; Thi et al. 2004). Jollivet et al. (2004) used intra-striatal glial cell line-derived neurotrophic-factor-releasing microspheres. Yasuhara et al. (2005) reported the use of glial cell line-derived neurotrophin-factor-producing cells encapsulated into hollow fibers. Kojima et al. (1997) and Cheng et al. (1998) found that glial cell-line-derived neurotrophic factor protects against MPTP-induced neurotoxicity in mice. Kordower et al. (2000) reported that neurodegeneration was prevented by lentiviral vector delivery of GDNF in

primate models (**rhesus monkeys** treated with MPTP) of Parkinson's disease.

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E.8 Animal Models of Neurological Disorders

E.8.1 Huntington's Disease

E.8.1.1

General Considerations

PURPOSE AND RATIONALE

Huntington's disease is a neurological disorder characterized by loss of striatal neurons and the motor signs of dyskinesia, dystonia and chorea, as well as complex neuropsychiatric changes (Hayden 1981). Brouillet et al. (1999) reviewed the different aspects of the replicating Huntington's disease phenotype in experimental animals. There is at present no effective therapy against this disorder. The gene responsible for the disease has been cloned and the molecular defect identified as an expanded polyglutamine tract in the N-terminal region of a protein, named huntingtin (Landles and Bates 2004; Li and Li 2004). Huntingtin interacts with a number of proteins and it has been suggested that alterations in glycolysis, vesicle trafficking or apoptosis play a role in the pathophysiology of Huntington's disease.

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E.8.1.2

3-Nitropropionic Acid Animal Model of Huntington's Disease

PURPOSE AND RATIONALE

An animal model of **Huntington's disease** was recommended by Beal et al. (1993), Brouillet et al. (1993), and Borlongan et al. (1995, 1997a, 1997b). Administration of 3-nitropropionic acid, an inhibitor of the

mitochondrial citric acid cycle, produces a very selective striatal degeneration and results in a progressive locomotor deterioration in rodents resembling that of Huntington's disease.

PROCEDURE

Borlongan et al. (1995) injected male Sprague Dawley rats i.p. with 10 mg/kg of 3-nitropropionic acid once every 4 days for 28 days and measured passive avoidance in a step-down apparatus and locomotor activity using the Digiscan Animal Activity Monitor System. Using the same system, Borlongan et al. (1997b) found hyperactivity after two injections, whereas four injections or more lead to hypoactivity.

Lee et al. (2000) evaluated the neuroprotective effect of lamotrigine and MK-801 on rat brain lesions induced by 3-nitropropionic acid by magnetic resonance imaging and *in vivo* proton magnetic resonance spectroscopy.

Male Sprague Dawley rats received daily i.p. injections of 15 mg/kg of 3-nitropropionic acid for five consecutive days. Test drugs were injected daily before 3-nitropropionic acid injections.

Behavioral changes were recorded daily, and immediately before sacrifice, graded according to the neurological scale described by Guyot et al. (1997). A total of 6 grades included: Grade 0, normal behavior; grade 1, general slowness in movement due to mild hind-limb impairment; grade 2, prominent gait abnormality with poor coordination; grade 3, nearly complete hind-limb paralysis; grade 4, incapability to move due to four-limb impairment; and grade 5, recumbency.

Magnetic resonance measurements were performed on the sixth day under anesthesia with 60 mg/kg i.p. pentobarbital. An endotracheal tube was set for artificial ventilation with an animal ventilator. The expiratory CO₂ concentration was maintained between 3.5% and 4.5% by adjusting the ventilator. Magnetic resonance measurements were performed on a Biospec 4.7 T spectrometer with an active shielding gradient at 6.9 Gy/cm in 500 μs. The rats were placed in a prone position with a custom-designed head-holder. A 20-cm birdcage coil was used for RF excitation, and a 2-cm-diameter surface coil placed directly over the head was used for signal receiving. After magnetic field optimization, a multi-slice multi-echo image was obtained with the following parameters: field of view = 5 cm, four slices (2 mm thick with a 1-mm gap), matrix 256 × 128, TR = 4,000 ms, and initial TE = 20 ms with an echo spacing of 20 ms for six echos. Pixel-by-pixel T2 maps were obtained

with commercial analysis software. The average of T2 values of bilateral hippocampus or striatum calculated from multi-echo images was applied for statistical analysis.

The point-resolved spectroscopy (PRESS) sequence preceded by three consecutive chemical shift selective saturation (CHESS) pulses for water suppression was used to acquire the localized proton spectra over the striatum. After manually adjusting the transmitter and receiver, magnetic field shimming of the striata and maximizing the suppression of the water signal, spectral data were obtained with the following parameters: TR = 2000 ms, TE = 136 ms, scan no. = 256, spectral width = 4000 Hz. The peak areas of *N*-acetyl-aspartate, choline, creatine, succinate, and lactate were recognized. The ratios of cerebral metabolites relative to creatine were used for statistical analysis.

EVALUATION

The data were expressed as means \pm SD. One-way ANOVA followed by a Tukey test was used for evaluating significant difference among sham controls and various treatment groups.

MODIFICATIONS OF THE METHOD

Matthews et al. (1998) found neuroprotective effects of creatine and cyclocreatine against lesions induced by malonate or 3-nitropropionic acid.

Quary et al. (2000) demonstrated major strain differences in response to chronic systemic administration of the mitochondrial toxin 3-nitropropionic acid in rats.

Pubill et al. (2001) reported that orphenadrine (an anticholinergic drug with NMDA receptor antagonist properties) prevented 3-nitropropionic-acid-induced neurotoxicity *in vitro* and *in vivo*.

Lastres-Becker et al. (2003) found that compounds acting on the endocannabinoid and/or endovanilloid systems reduce hyperkinesia in rats induced by bilateral intrastriatal injection of 3-nitropropionic acid.

Lee and Chang (2004) used magnetic resonance imaging and spectroscopy to assess 3-nitropropionic-acid-induced brain lesions in rats.

Tunéz et al. (2004) described a protective effect of melatonin on 3-nitropropionic-acid-induced oxidative stress in synaptosomes in rats.

Hantraye et al. (1992) reported that intrastriatal transplantation of cross-species fetal striatal cells reduced abnormal movements in **baboons** injected with the neurotoxin ibotenic acid.

Palfi et al. (1996) showed that chronic 3-nitropropionic acid treatment replicates the cognitive and motor deficits in Huntington's disease also in **baboons**.

Mittoux et al. (2000) reported restoration of cognitive and motor function by ciliary neurotrophic factor in a primate model (*Macaca fascicularis*) of Huntington's disease.

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E.8.1.3

Quinolinic Acid Rat Model of Huntington's Disease

PURPOSE AND RATIONALE

Intrastriatal injection of quinolate, an NMDA receptor agonist, replicates many neurochemical, histological, and behavioral features of Huntington's disease (Beal et al. 1986, 1988, 1991; DiFiglia 1990; Burns et al. 1995; Pérez-Navarro et al. 2000a).

Araujo and Hilt (1997) found that the glial cell-line-derived neurotrophic factor attenuates the behavioral and neurochemical deficits induced by quinolinic acid in rats.

PROCEDURE

Female Sprague Dawley rats were anesthetized with pentobarbital (50 mg/kg) before being placed in a stereotaxic apparatus (Kopf). A small hole was made on the left side of the skull 3.0 mm lateral to the midline, through which a hypodermic needle attached to a Hamilton syringe was lowered 5 mm below the dura into the striatum. For each lesioned rat, 200 nmol of quinolinic acid dissolved in 2 μ l of phosphate-buffered saline was injected over a 1-min interval through the needle into two sites (0.7 and 1.4 mm anterior to the bregma) in the left striatum, while the contralateral striatum was left intact.

Neurotrophic factors were given 30 min prior to the toxin injections. Recombinant glia-cell-derived neurotrophic factor (Lin et al. 1993; Lapchak et al. 1997), brain-derived neurotrophic factor or neurotrophin-3 was administered intraventricularly. For i.c.v. treatment, a Hamilton syringe or an infusion cannula was lowered through a hole drilled in the skull of anesthetized rats at the following coordinates: 1.4 mm posterior to the bregma, 2 mm lateral to the midline, and 3.5 mm below the dura.

Rotational Behavior

The effects of quinolinic acid lesions and neurotrophic factor treatment on rotational behavior were assessed using a Rota-Count 8 automated rotor system (Columbus Instruments), which monitors both clockwise and counterclockwise turning behavior. One week following surgery, rats were given 5 mg/kg amphetamine i.p. 15 min prior to placement into the apparatus. The total number of turns in successive 10-min intervals was measured in a 1-h period.

Histology

The extent of the lesion produced by quinolinic acid was illustrated using Nissl-stained coronal sections.

D₁ and D₂ dopamine receptor binding was evaluated in homogenized lesioned and unlesioned striata.

Striatonigral [³H]neurotransmitter uptake was measured in synaptosomes from striatal tissue containing putamen-pallidum as well as a portion of the globus pallidus.

Striatal choline acetyltransferase activity was determined in striatal homogenates using a radiochemical assay.

Neuropeptide levels in tissue homogenates were measured using commercial radioimmunoassay kits.

EVALUATION

Results are expressed as mean \pm SEM of the number of rats, where each sample was the average of duplicate or triplicate measures. Statistical significance was assessed using a one-way ANOVA, followed by post hoc Bonferroni or Dunnett's Multiple Comparisons analysis.

MODIFICATIONS OF THE METHOD

Anderson et al. (1996) reported that the ciliary neurotrophic factor protects striatal output neurons in the quinolinic acid rat model.

Nakao et al. (1996) studied paw reaching ability in rats with unilateral quinolinic acid lesions of the striatum.

Shear et al. (1998) compared intrastriatal injections of quinolinic acid and 3-nitropropionic acid for use in animal models of Huntington's disease.

Hughes et al. (1999) found that administration of recombinant human activin-A has powerful neurotrophic effects on selected striatal phenotypes in the quinolinic acid lesion model.

Pérez-Navarro et al. (1999) reported that intrastriatal grafting of a glia cell-line-derived neurotrophic factor-producing cell line protects striatonigral neurons from quinolinic acid excitotoxicity *in vivo*.

Reggio et al. (1999) found that the intrastriatal injection of an adenosine A₂ receptor antagonist prevents frontal cortex EEG abnormalities in the quinolinic acid rat model.

Nakao et al. (1999) reported that embryonic striatal grafts restore neuronal activity of the globus pallidus in the quinolinic acid rat model.

Pérez-Navarro et al. (2000b) found that neurturin protects striatal neurons but not interneurons in the quinolinic acid rat model.

Bensadoun et al. (2001) found a neuroprotective effect of interleukin-6 and IL6/IL6R chimera in the quinolinic acid rat model.

Gianfriddo et al. (2003) found that adenosine A_{2A} antagonism increases striatal glutamate outflow in the quinolinic acid rat model.

Ryu et al. (2003) described neuroprotective effects of pyruvate in the quinolinic acid rat model.

Scattoni et al. (2004) reported progressive behavioral changes in the spatial open-field in the quinolinic acid rat model.

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E.8.1.4

Transgenic Animal Models of Huntington's Disease

PURPOSE AND RATIONALE

Several **transgenic models** of Huntington's disease are described, among them:

The **R6/2 HD mouse model** (Ona et al. 1999; Hickey and Morton 2000; Hemlinger et al. 2002; Keene et al. 2002; Hockly et al. 2003a, 2003b; Zucker et al. 2004; Chou et al. 2005);

the **yeast artificial chromosome (YAC) mouse** (Ainscough et al. 2001; Slow et al. 2003; Al Mahdawi et al. 2004);

the **TgCAG100 mouse** (Laforet et al. 2001; Ariano et al. 2005);

the **Hdh^{Q111} mouse** (Gines et al. 2003).

A **transgenic rat model** of Huntington's disease was described by Von Hörsten et al. (2003). Behavioral phenotyping was performed using computerized systems from TSE Systems (Bad Homburg, Germany).

Segalat and Neri (2003) recommended *Caenorhabditis elegans* as a model for human inherited degenerative disorders, such as Duchenne's muscular dystrophy and Huntington's disease.

A **transgenic *Drosophila* model** of Huntington's disease was recommended by Lee et al. (2004) and Agrawal et al. (2005).

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E.8.2

Amyotrophic Lateral Sclerosis

E.8.2.1

General Considerations

PURPOSE AND RATIONALE

Amyotrophic lateral sclerosis is an adult-onset neurological disease characterized by the selective loss of motor neurons. The early symptoms, principally limb and bulbar muscle weakness, begin in middle life and progress usually rapidly to death within 2 or 3 years. Clinical subtypes of the disease are defined according to the predominant site of weakness and whether upper or lower motor neurons are primarily involved (Rowland and Shneider 2001; Morrison 2002; Waldmeier 2003). Several experimental models for amyotrophic lateral sclerosis have been developed (Kaal et al. 1999; Anger 1991; Senior 2002).

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E.8.2.2**Transgenic Animal Models of Amyotrophic Lateral Sclerosis****PURPOSE AND RATIONALE**

Most of the transgenic mouse models are based on familial superoxide dismutase 1 (SOD1) mutations. Of these, the **G93A** mouse is the most investigated (Gurney et al. 1994; Kostic et al. 1997; Trieu and Uckun 1999; Vukosavic et al. 1999, 2000; Azzouz et al. 2000; Li et al. 2000; Spooen and Hengerer 2000; Andreassen et al. 2001; Canton et al. 2001; Jung et al. 2001; Wendt et al. 2002; Azari et al. 2003; Copray et al. 2003; Maragakis et al. 2003; Snow et al. 2003; van Damme et al. 2003; Raiteri et al. 2004; Raman et al. 2004; Waibel et al. 2004). These mice express mutant human SOD1 with a substitution of glycine to alanine in position 93, which induces a severe, progressive motoneuron disease. Several studies were performed with **G37R** (Nguyen et al. 2001; Farah et al. 2003; Robertson et al. 2003, 2003), **H46R** (Nagai et al. 2001), and **G85R** (Bruijn et al. 1998; Amedola et al. 2004) mutant mice. Pasinelli et al. (1998) reported cleavage and activation of caspase-1 in these animals, and Lee et al. (2001) found increased vulnerability of cells transfected with these mutant forms of SOD1 to apoptotic stimuli and protection by overexpression of Bcl-2.

Van Damme et al. (2003) tested the AMPA receptor antagonist NBXQ for survival in a transgenic mouse model of amyotrophic lateral sclerosis. Canton et al. (2001) described the pharmacological properties and activity in the SOD1-G53A animal model of amyotrophic lateral sclerosis of a synthetic AMPA antagonist.

PROCEDURE

Transgenic mice [B6SJL-TgN(SOD1-G93A)G1H] heterozygous for the deficient SOD1 gene and wild-

type littermates were identified by polymerase chain reaction. Animals were treated starting on the 50th day of life, until their death with various subcutaneous doses of the test compound.

The following parameters were examined:

Muscle strength: Animals were tested for muscle strength at intervals using a muscle strength meter. Each mouse was held by the tail and pulled steadily over a metal grill three times in rapid succession. The force was recorded on a force meter. The highest score for muscle strength was taken.

Glutamate uptake: Animals were sacrificed at 150 days and spinal cord rapidly dissected for synaptosomal preparations and high-affinity Na⁺-dependent glutamate uptake was measured. Spinal cords were homogenized in 20 volumes of sucrose (0.32M) and the homogenate centrifuged at 800 g for 10 min. After centrifugation of the supernatant at 20,000 g for 20 min, the pellet was resuspended and washed in 50 volumes of sucrose buffer. Transport assays were performed in Krebs-HEPES buffer. Duplicate samples, with or without sodium chloride, were incubated at 37°C for 3 min in the presence of [³H]L-glutamate after appropriate isotopic dilution. The reaction was stopped by addition of ice-cold buffer containing choline and followed by filtration and scintillation counting of the radioactivity retained on filters. High-affinity Na⁺-dependent uptake was calculated by subtracting results obtained in choline buffer from that obtained with Na⁺ buffer.

Life expectancy: Remaining mice were treated until day of death, which was noted.

EVALUATION

Statistical significance of the biochemical and behavioral data was assessed using Kruskal-Wallis non-parametric analysis of variance followed by Dunn's multiple comparison test.

MODIFICATIONS OF THE METHOD

Haase et al. (1998) described therapeutic effects and mechanism of action of adenovirus-mediated transfer of the neurotrophin-3 gene into skeletal muscle of pnm (progressive motor neuropathy) mice.

Schmalbruch et al. (1991) described an autosomal-recessive mouse mutant with progressive motor neuropathy. Homozygotes develop paralysis of the hindlimbs during the third week of life. Soon thereafter the forelimbs also become weak, and all mice die 6–7 weeks after birth.

Zhu et al. (2002) reported that minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in transgenic mice.

Howland et al. (2002) described focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis.

Bordet et al. (1999, 2001) reported protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic amyotrophic lateral sclerosis mice overexpressing a mutated form of human superoxide dismutase-1 with a Gly⁹³Ala substitution.

Ralph et al. (2005a, 2005b) reported that a silencing mutant SOD1 using interfering RNA protects against neurodegeneration and extends survival in the G93A model of amyotrophic lateral sclerosis.

Pharmacology and mechanism of action of riluzole, which has demonstrated beneficial effects in amyotrophic lateral sclerosis in clinical studies, have been investigated in several preclinical studies (Doble 1996; Gurney et al. 1998; Jimonet et al. 1999; Snow et al. 2003).

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E.8.3

Spinal Muscular Atrophy

E.8.3.1

General Considerations

PURPOSE AND RATIONALE

Spinal muscular atrophy is a common genetic disease of the motor neuron with a high mortality during infancy (Strober and Tennekoon 1999; Iannaccone et al. 2004; Ogino and Wilson 2004). Among the group of clinically and genetically heterogeneous spinal muscular atrophies, the autosomal recessive proximal types I–III are the most frequent (Schara and Mortier 2004). They are caused by mutations of the telemetric copy of the survival motor neuron gene (SMN1) on chromosome 5q while loss of the centromeric copy (SMN2) does not lead to spinal muscular atrophy (Talbot and Davis 2001; Frugier et al. 2002; Wirth 2002; Nicole et al. 2002).

Transgenic mouse models have been developed for these types of disease.

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E.8.3.2

Transgenic Animal Models of Spinal Muscular Atrophy

PURPOSE AND RATIONALE

Several mouse models of spinal muscular atrophy are described (Bergin et al. 1997; Deniselle et al. 1997;

DiDonato et al. 1997, 2001; Schrank et al. 1997; Fricker 2000; Frugier et al. 2000; Hsieh-Li et al. 2000; Jablonka et al. 2000, 2001; Monani et al. 2000a, 2000b, 2003; Cifuentes-Diaz et al. 2001, 2002; Melki 2001; Tucker et al. 2001; Ferri et al. 2003, 2004; Rossoll et al. 2003; Azzouz et al. 2004; Grohmann et al. 2004; Le et al. 2005). These studies are intended to mimic spinal muscular atrophy in humans resulting from loss-of-function mutations in the survival motor neuron gene (*Smn*). Mice carrying a homozygous deletion of *Smn* exon 7 display a dramatic and progressive loss of motor axons involving both proximal and terminal regions. The human centromeric survival motor neuron gene (*SMN2*) rescues embryonic lethality in *Smn*^{-/-} mice. SMN Δ 7, the major product of the centromeric survival motor neuron (*SMN2*) gene, extends survival in mice with spinal muscular atrophy.

Chang et al. (2001) reported treatment of spinal muscular atrophy in transgenic mice by sodium butyrate.

PROCEDURE

Cell Culture

Epstein-Barr-virus-transformed lymphoid cell lines from different SMA-type patients with deleted *SMN1* genes were established. Lymphocytes were collected from the whole blood of patients by Ficoll hypaque separation. The buffy coat was collected and washed twice with 5 ml PBS. The pellet was resuspended in 5 ml RPMI medium containing 0.5 ml Epstein-Barr virus, 50 μ l phytohemagglutinin (0.5 mg/ml), and 50 μ l ciclosporin (0.2 mg/ml). Cells were incubated at 37°C with 5% CO₂ until they became viable.

Mice

Five independent human *SMN2* gene transgenic mice were generated and crossed with mice heterozygous for the *Smn* locus knockout. Transgenic mice that were also homozygous for the knockout alleles (*Smn*^{-/-}*SMN2*) were then generated by crossing with the above mice. These knockout transgenic mice developed progressive motor neuron disease similar to that in human SMA patients. The SMA-like mice were classified into three groups based on their phenotypes, which were judged by three independent observers. Mice with the most severe pathological form (type 1) did not develop furry hair and died before postnatal day 10; mice with intermediate severity (type 2) showed poor activity and variable symptoms and died at 2–4 weeks; the type 3 mice survived and bred normally, but had short and enlarged tails (Hsieh-Li et al. 2000). SMA-like mice (non-pregnant and pregnant)

were supplied with sterile water ad libitum and rodent pellets. The sodium butyrate-treated group received sodium butyrate at a concentration of 0.8 mg/ml or 8 mg/ml in distilled water, beginning immediately after diagnosis or after 15 days gestation in SMA-like pregnant mice. Both groups consumed about 5–10 ml per day per mouse. After 1–12 weeks of treatment, the mice were sacrificed, and their organs or tissues were quickly removed and frozen in liquid nitrogen.

Reverse Transcriptase-PCR Analysis

Reverse transcriptase-PCR analysis was performed according to Hsieh-Li et al. (2000) and Jong et al. (2000).

Subcellular Fractionation

Fresh frozen spinal cord, brain, and skeletal muscle samples (500 mg) from different types of SMA mice were fractionated. Tissues were homogenized with a tight-fitting glass pestle in ice-cold buffer. The nuclei were pelleted by centrifugation at 800 *g* for 3 min. The nuclear pellet was resuspended by trituration in 100 μ l of buffer and kept on ice for 15 min followed by centrifugation at 15,000 *g* for 10 min at 4°C. The supernatant (soluble nuclear extract) was removed, and the insoluble nuclear pellet was further sonicated in sonication buffer.

Western Blot Analysis

Synthetic peptides containing part of human *SMN* exon 7 (amino acids 279–288) and exon 2 (amino acids 72–84) were used to immunize rabbits and to purify specific antibodies (H2 and H7) from rabbit crude sera with an EAH Sepharose 4B column. Two mouse anti-SR protein antibodies (anti-SRp20 and 16H3) were used to detect the human SR proteins. Proteins were loaded on a 5% polyacrylamide stacking gel above a 12% separating gel, and the gel was run with a discontinuous buffer using Laemmli's method. After electrophoresis, proteins were transferred electrophoretically to poly-(vinylidene difluoride) membranes. After the transfer, the membranes were blocked with TBST containing 4% BSA for 2 h at room temperature. The blots were washed for three 20-min periods in TBST and then incubated with a 1:32,000 dilution of an anti-rabbit IgG alkaline phosphatase conjugate in TBST for 1 h at room temperature. The reaction was detected by adding 1.5% 5-bromo-4-chloro-3-indolyl phosphate and 3% nitro blue tetrazolium in a developing buffer.

Histopathological Analysis

Histopathological analysis was performed according to Hsieh-Li et al. (2000).

EVALUATION

Results from multiple experiments were expressed as mean \pm standard error. Survival data of treated and untreated mice are presented as a Kaplan-Meier plot using the log rank test. A standard χ^2 test was used to assess differences in the frequency of mild or severe phenotype in the SMA-like mice born from treated and untreated mothers, which analyzed the percentage of type 1 (or 2+3) newborn mice as a fraction of the total number of pups.

MODIFICATIONS OF THE METHOD

Lesbordes et al. (2003) reported therapeutic benefits of cardiotoxin-1 gene transfer in a mouse model of spinal muscular atrophy.

Andreassi et al. (2001) found that aclarubicin treatment restored SMH levels to cells derived from type I spinal muscular atrophy patients.

Haddad et al. (2003) reported that riluzole attenuates spinal muscular atrophy disease progression in a mouse model.

He et al. (2005) described an inherited motor neuron disease in domestic cats as a model of spinal muscular atrophy.

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E.8.4

Spinal and Bulbar Muscular Atrophy

E.8.4.1

General Considerations

PURPOSE AND RATIONALE

Spinal and bulbar muscular atrophy is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. The disease exclusively affect males, while females are usually asymptomatic (Katsuno et al. 2003, 2004).

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E.8.4.2

Transgenic Animal Models of Spinal and Bulbar Muscular Atrophy

PURPOSE AND RATIONALE

Spinal and bulbar muscular atrophy is a polyglutamine disease caused by expansion of a CAG repeat in the androgen receptor gene (Adachi et al. 2001; Katsuno et al. 2002; Walcott and Merry 2002). Several trans-

genic mouse models were developed (Bates and Davies 1997; Abel et al. 2001; MacManamy et al. 2002).

Katsuno et al. (2003) reported that leuprolin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy.

PROCEDURE

Animals

Transgenic mice were generated carrying a full-length androgen receptor containing 97 CAGs (Katsuno et al. 2002). A full-length human androgen receptor fragment was subcloned containing 24 or 97 CAG repeats (Kobayashi et al. 1998) into a pCAGGS vector (Niwa et al. 1991) digested with *Hind*III. The result was microinjected into fertilized eggs of BDF1 mice. Five founders with AR-97Q were obtained. These mouse lines were maintained by back-crossing with CS7B1/6 mice.

Neurological status was measured with the Rotarod test (Columbus Instruments) and cage activity with the AB system (Neuroscience, Tokyo).

Hormonal Treatment

Leuprolin acetate was injected subcutaneously at a dose of 25, 50 or 100 μ g per mouse every 2 weeks from 5 weeks of age. Control mice were treated with vehicle. Leuprolin-treated AR-97Q mice were given either leuprolin only or leuprolin plus 20 μ g testosterone enanthate dissolved in sesame oil s.c. weekly from the age of 13 weeks. Flutamide was injected at a dose of 1.8 mg per mouse once every second day.

Serum testosterone was assayed with a radioimmunoassay.

Protein Expression Analysis

Mice were sacrificed by anesthesia and exsanguination and their tissues snap-frozen with powdered CO₂ in acetone. Tissues were homogenized in 50 mM TRIS, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% SDS and 1 mM 2-mercaptoethanol with 1 mM PMSF and 6 μ g/ml aprotinin and centrifuged at 2,500 g for 15 min at 4°C. Each lane of a 5%–20% SDS-PAGE gel was loaded with 160 μ g protein from nerve tissue and 80 μ g for muscle from the respective supernatant fraction. This was transferred on Hybond-P membranes in a transfer buffer of 25 mM TRIS, 192 mM glycine, 0.1% SDS and 10% methanol. After immuno-probing with a rabbit antibody to the androgen receptor, a secondary antibody probing and detection with the ECL Plus kit (Amersham) was performed. The signal intensity of the bands was quantified using the NIH Image program. The quantita-

tive data of three independent Western blots were expressed as mean \pm SD.

Immunohistochemistry and Histology

Deeply anesthetized mice were perfused through the left cardiac ventricle with 20 ml of a 4% paraformaldehyde fixative in phosphate buffer. The tissues were post-fixed overnight in 10% phosphate-buffered formalin and processed for paraffin embedding. De-paraffinized tissue sections 4 μ m in thickness were dehydrated in alcohol, treated with formic acid for 5 min at room temperature and stained with the polyglutamine-specific antibody 1C2 (1:10,000 dilution) (Holmberg et al. 1998). After formalin fixation, tail specimens were washed with 70% alcohol and decalcified with 7% formic acid and 70% ethanol for 7 days followed by paraffin embedding. To assess 1C2-positive cells in muscle, the number of 1C2-positive cells in more than 500 fibers in the entire area was calculated and expressed as the number per 100 muscle fibers. The air-dried cryostat sections (6 μ m in thickness) of gastrocnemius muscles were stained with hematoxylin-eosin.

EVALUATION

Data were analyzed using the unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Adachi et al. (2003) reported that heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular transgenic mouse model by reducing nuclear localized mutant androgen receptor protein.

Chevalier-Larsen et al. (2004) found that castration restores the function and neurofilament alterations of aged symptomatic males in a transgenic mouse model of spinal and bulbar muscular atrophy.

Minamiyama et al. (2004) reported that sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy.

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E.8.5

Models of Down Syndrome

PURPOSE AND RATIONALE

Down syndrome, the most common known genetic cause of mental retardation, is the result of trisomy of chromosome 21. Although the degree of cognitive impairment in Down syndrome can be quite variable, individuals with Down syndrome have significantly lower IQs with impaired speech and language skills as well as specific difficulties in auditory and visual memory, in spatial memory and in the acquisition of conditioned and operant responses. Down syndrome is considered to be caused by trisomy of the human chromosome 21.

Several attempts have been made to find animal models for Down disease. Since the mouse chromosome 16 is homologous to the human chromosome 21, mice with trisomy of chromosome 16 were created. Cox et al. (1984) reported production of a viable trisomy 16 diploid mouse chimera as a model of human trisomy 21 (Down syndrome). This model has been extensively used (Epstein et al. 1985; Sweeney et al. 1989; Holtzman et al. 1992; Shetty et al. 1996). However, the value of this model is limited to some extent because trisomy 16 mouse fetuses do not survive as live-born animals.

The **Ts65Dn mouse** carries only a partial trisomy for mouse chromosome 16 in a region that has highly homology to the Down syndrome region of human chromosome 21. These mice survive until adulthood and have therefore been used as animal model for Down syndrome in more recent studies (Davisson et al. 1993; Coussons-Read and Crnic 1996; Klein et al. 1996; Kola and Hertzog 1997; Costa et al. 1999; Cooper et al. 2001; Galdzicki et al. 2001; Granholm et al. 2002; Bimonte-Nelson et al. 2003; Stasko and Costa 2004).

Ema et al. (1999) described an animal model of Down syndrome in mice overexpressing the *mSim2* gene located on chromosome 16. Borella et al. (2003) characterized social behaviors and oxytoninergic neurons in the S-100 β overexpressing mouse model of Down syndrome originally described by Friend et al. (1992).

A number of experimental compounds have been developed that result in improved cognitive performance in animal models. Moran et al. (2002) described the effects of piracetam on cognitive performance in a mouse model of Down syndrome.

PROCEDURE

Male 5-week-old Ts65Dn and euploid littermate control mice received daily injection of one of three dose of piracetam (75, 150, and 300 mg/kg) or saline vehicle. Piracetam treatment continued for 4 weeks prior to and throughout the 4-week testing period. Testing consisted of assessments of performance on the visible- and hidden-platform components of the Morris maze task and assessment of spontaneous activity within computerized activity chambers.

EVALUATION

Significant effects were explored with two-way ANOVA, analysis of main effects, and paired *t* comparisons.

MODIFICATIONS OF THE METHOD

Cardenas et al. (2002) established and characterized immortal neuronal cell lines derived from the spinal cord of normal and trisomy 16 fetal mice.

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E.8.6

Models of Wilson's Disease

PURPOSE AND RATIONALE

Wilson disease is an **inherited disorder of copper metabolism** caused by autosomal recessive mutations of the *ATP7B* gene. The mutant *ATP7B* proteins lead to an impaired copper efflux, a decreased copper incorporation into ceruloplasmin and to hepatic copper accumulation, resulting in hepatocellular injury and deposition of copper in extra-hepatic tissues. Patients suffer from chronic or acute liver disease, psychiatric symptoms and neurological abnormalities with extrapyramidal symptoms, such as tremor and ataxia. D-penicillamine has been used for treatment of patients with Wilson disease, but more recently ammonium tetrathiomolybdate has been recommended (Stremmel et al. 1991; Silva et al. 1996; Brewer et al. 2003).

The **Long-Evans Cinnamon rat** has a mutation homologous to the human Wilson disease gene, leading to gross copper accumulation and development of hepatitis. This rat strain has been extensively used as animal model for Wilson disease (Yamaguchi et al. 1994; Suzuki 1995; Adachi et al. 1997; Kodama et al. 1998; Nagano et al. 1998; Nomiya et al. 1999; Terada and Sugiyama 1999; Klein et al. 1998, 2000, 2004; Komatsu et al. 2000, 2002; Meng et al. 2004).

Klein et al. (2004) reported the effects of tetrathiomolybdate in the treatment of acute hepatitis in Long-Evans Cinnamon rats.

PROCEDURE

Female Long-Evans Cinnamon rats (Charles River, Japan) at an age of between 60 and 100 days were used, whereas female Long-Evans Aguti rats served as controls. Long-Evans Cinnamon (LEC) rats were randomly divided in different groups:

LEC rats without any treatment and without signs of liver disease,

jaundiced LEC rats without any treatment,

jaundiced LEC rats treated once intraperitoneally with 10 mg/kg tetrathiomolybdate.

The tetrathiomolybdate-treated rats were sacrificed either 1 or 4 days after treatment.

Tissue Preparation and Subcellular Fractionation

Livers were flushed *in situ* with ice-cold 0.9% NaCl in order to remove the blood. A part of the liver was fixed in 4% neutral formalin, embedded in paraffin, and sections were stained for light microscopy. From homogenates lysosomal and mitochondrial fractions were prepared (Klein et al. 1998, 2000). Electron microscopy and X-ray microanalysis were performed. Metals and metallothionein were quantified.

EVALUATION

Student's *t*-test was used for statistical evaluation.

OTHER DISORDERS OF COPPER METABOLISM

Besides Wilson's syndrome, other inherited diseases of cellular copper metabolism are known, such as Menkes disease and the toxic milk syndrome in mice (Palida and Ettinger 1991; La Fontaine et al. 2001).

Menkes' syndrome is an X-linked recessive disorder associated with copper deficiency, lethal in early childhood. Menkes' children show impaired synthesis of collagen and elastin. Furthermore, they are severely mentally retarded. Diffuse atrophy, focal degeneration of gray matter and prominent neuronal loss have been detected in the cerebellum. The molecular basis of this syndrome has been identified as a mutated gene (*ATP7A*) encoding for a P-type cation-transporting ATPase (Kodama and Murata 1999; Tumer et al. 1999; Rossi et al. 2001).

The mutant **macular** mouse has been used as animal model for Menkes' disease (Tanaka et al. 1990; Murata et al. 1997, 1998; Suzuki-Kurasaki et al. 1997). The **brindled mouse mutant** (*Mo^{br}*) is considered to be the closest model to Menkes' disease (Grimes et al. 1997; Rossi et al. 2001). The mutant mice are hypopigmented and die at around 15 days after birth, but can be saved by treatment with copper before the 10th postnatal day.

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E.8.7

Models of Cerebellar Ataxia

The pathology mechanisms underlying the signs of hereditary cerebellar ataxia in humans are not well understood (Harding 1984).

Kato et al. (1982) and Yamaguchi et al. (1992) regarded the rolling mouse Nagoya as an animal model of hereditary cerebellar ataxia.

Niimi et al. (1982) made an animal model of cerebellar ataxia by injections of kainic acid into both sides of the cerebellar hemispheres of male rats.

Grüsser-Cornehls et al. (1999) recommended Weaver mutant mice as an animal model of cerebellar ataxia.

Fernandez et al. (1998) induced ataxia in adult rats either with intraperitoneal injection of 50–65 mg/kg 3-acetylpyridine or by bilateral electrocoagulation of the inferior olive. Insulin-like growth factor 1 restored motor coordination in this rat model of cerebellar ataxia.

Ataxia-telangiectasia is a complex multiparametric disease associated with oculocutaneous telangiectasias, cerebellar ataxia, elevated chromosomal aberration frequency, and varied degrees of immunodeficiency.

ciency. Kaiserlian et al. (1986) described the wasted mouse as a model of this disease.

Clark et al. (2000) reported X-linked transmission of the *shaker* mutation in rats with hereditary Purkinje cell degeneration and ataxia.

Sausbier et al. (2004) described cerebellar ataxia and Purkinje cell dysfunction in mice caused by Ca^{2+} -activated K^{+} channel deficiency

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E.8.8

Models of Niemann-Pick Syndrome

PURPOSE AND RATIONALE

Niemann-Pick disease type C is a progressive neurodegenerative disorder caused by mutations in the *NPC1* gene and characterized by intracellular accumulation of cholesterol and sphingolipids.

Pentchev et al. (1980, 1984) described a genetic storage disorder in BALB/C mice with metabolic block in esterification of exogenous cholesterol. The murine model of Niemann-Pick C disease with mutation in a cholesterol homeostasis gene (*npc1*^{-/-} mutant mice) has been used in several stud-

ies (Weintraub et al. 1985, 1987; Higashi et al. 1993; Loftus et al. 1997; Erickson et al. 2000; Bascunan-Castillo et al. 2004). Some attempts have been made to improve the clinical course of the Niemann-Pick C disease mouse (Liu et al. 2000). Treatment with cyclodextrins had variable effects in delaying neurological symptoms and in decreasing liver cholesterol storage (Camargo et al. 2001). Zhan et al. (2004) showed that cyclin-dependent kinase inhibitors attenuate protein hyperphosphorylation, cytoskeletal lesion formation, and motor defects in Niemann-Pick type C mice.

MODIFICATIONS OF THE METHOD

Sym et al. (2000) described a model for Niemann-Pick type C disease in the nematode *Caenorhabditis elegans*.

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E.8.9

Models of Gangliosidosis

PURPOSE AND RATIONALE

The glycosphingolipid lysosomal storage diseases are inherited metabolic diseases in which a gene encoding a lysosomal hydrolase or one of their co-factors is mutated. Two classes of glycosphingolipid storage diseases are known, those in which the storage glycosphingolipid is neutral (**Gaucher** and **Fabry** disease) and the gangliosidoses in which the storage lipid is a ganglioside (glycosphingolipid containing one or more sialic acid residues). The gangliosidoses include the GM₂ storage disorders, **Tay-Sachs** and **Sandhoff disease** (O'Dowd et al. 1986; Paw et al. 1989; Koller and Sandhoff 1998; Schröder et al. 1991; Glaros et al. 2005), and the GM₁ storage disorder termed GM₁ gangliosidosis or **Morquio B** disease (Matsuda et al. 2003).

Spontaneous gangliosidosis has been described in several animal species, such as **dogs** (Singer and Cork 1989; Kaye et al. 1992; Wang et al. 2000; Yamato et al. 2003, 2004b; Kreutzer et al. 2005), **cats** (Cork et al. 1977; Neuwelt et al. 1985; Muldoon et al. 1994; Kroll et al. 1995; Yamato et al. 2004a; Martin et al. 2005), **sheep** (Skelly et al. 1995; Ryder and Simmons 2001), **deer** (Fox et al. 1999), and **pigs** (Kosanke et al. 1978).

β -Galactosidase-deficient mice were described as an animal model for GM₁-gangliosidoses (Hahn et al. 1997; Matsuda et al. 1997a, 1997b; Oshima 1998; Callahan 1999; Itoh et al. 2001). Mouse models of GM₂ gangliosidosis are reported by Huang et al. (1997) and Jeyakumar et al. (2003).

No effective treatment of patients is known as yet, but Jeyakumar et al. (1999) found delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with *N*-butyldeoxynojirimycin. Yamaguchi et al. (2003) reported that plasmid-based gene transfer ameliorates visceral storage in a mouse model of Sandhoff disease. Martino et al. (2002) described restoration of the GM₂ ganglioside metabolism in bone-marrow-derived stromal cells from a Tay-Sachs

disease mouse model. Furthermore, Martino et al. (2005) reported that a direct gene strategy via brain internal capsule reverses the biochemical defect in Tay-Sachs disease.

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E.8.10

Models of Mucopolysaccharidosis

PURPOSE AND RATIONALE

Mucopolysaccharidosis type IIIA or **Sanfilippo syndrome** is a lysosomal storage disorder characterized by progressive neurological pathology. Patients exhibit aggression, disturbed sleep, hyperactivity and mental decline ultimately resulting in inanition and death. Four mucopolysaccharidosis subtypes result from deficiencies in different lysosomal enzymes that sequentially degrade heparin sulfate: sulfamidase (MPS IIIA), α -N-acetylglucosaminidase (MPS IIIB), acetyl-CoA: α -glucosaminidase N-acetyltransferase (MPS IIIC), and glucosamine-6-sulfatase (MPS IIID).

Mucopolysaccharidosis III has been described in several animal species, such as mice (Li et al. 1999, 2002; Yu et al. 2000; Gografe et al. 2003; Garbuzova-Davis et al. 2005), dogs (Fischer et al. 1998; Aronovich et al. 2000; Ellinwood et al. 2003), emus (Giger et al. 1997; Aronovich et al. 2001), and goats (Thompson et al. 1992).

A naturally occurring mouse model of MPS-IIIa has been discovered (Bhaumik et al. 1999; Bhattacharyya et al. 2001), with pathophysiology and symptoms that resemble the human conditions (Glid-

don and Hopwood 2004; Hemsley and Hopwood 2005).

No effective drug treatment for patients with Sanfilippo disease is available; however, experiments in mice showed beneficial effects after transplantation of human umbilical cord blood cells (Garbuzova-Davis et al. 2005), or treatment with lentiviral NAGLU vector (Di Natale et al. 2005) or enzyme replacement (Yu et al. 2000; Gliddon and Hopwood 2004).

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E.9 General Anesthesia

E.9.1 Intravenous Anesthesia

E.9.1.1 General Considerations

PURPOSE AND RATIONALE

The first agents which could be used as intravenous anesthetics were **barbiturates**. Barbiturates with a duration of action appropriate to the requirements of surgery became available with the introduction of hexobarbital and thiopental (Volwiler and Tabern 1930; Miller et al. 1936). The studies with barbiturates were extended (Butler and Bush 1942; Christensen and Lee 1973). Intravenous anesthetics from other chemical groups were developed, such as **acetamidoeugenol** (Estil, Domenjoz 1959), steroid derivatives (Presuren = **hydroxydione sodium**, Laubach et al. 1955; **alfaxolone**, CT1341, Child et al. 1971), **propanidid** (Epontol, Goldenthal 1971), **ketamine** (CI-581, Chen et al. 1966; Reich and Silvay 1989), **etomidate** (Janssen et al. 1975), **propofol** (ICI 35868, Glen 1980), **midazolam** (Pieri 1983; Reilly and Nimmo 1987).

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E.9.1.2

Screening of Intravenous Anesthetics

PURPOSE AND RATIONALE

Screening of intravenous anesthetics or hypnotics is performed mostly in mice or rats. Doses for loss of righting reflex and lethal doses are determined. Onset of action and duration of action are the secondary parameters.

PROCEDURE

Male mice weighing 18–22 g are injected intravenously via the tail vein. The anesthetic activity is estimated from the number of animals that lose their righting reflex. The righting reflex is considered lost when the mouse, placed on its back, fails to recover from this position within 1 min. The acute toxicity is based on lethality within a 24-h observation period.

To determine onset and duration of action groups of 20 mice are used. They are placed in individual observation cages maintained at room temperature (24 ± 1°C). They are not stimulated during the interval between loss and recovery of the righting reflex. The onset is defined as the complete loss of the righting reflex, i. e., no attempt to move the head or body. Recovery is considered to have occurred when the animal after

spontaneous righting would re-right itself within 15 s when placed on its back.

EVALUATION

The median anesthetic dose (AD₅₀) and the median lethal dose (LD₅₀) are determined from dose-response curves with at least 4 doses by the method of Litchfield and Wilcoxon (1949).

The data for onset and duration of action are analyzed statistically by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Volwiler and Tabern (1930) determined the minimum effective dose in rats after subcutaneous injection of various barbiturates not being awakened when outer ear passage was tickled with a straw.

Büch et al. (1968) studied the distribution, anaesthetic potency and metabolic elimination of the optical isomers of methylphenobarbital in rats.

Glen (1977) described a method for the laboratory evaluation of the speed of onset of i.v. anesthesia in mice. Various clinically used intravenous anesthetics were compared. The technique involves a) determination of the medium hypnotic dose (HD₅₀) by plotting the probit value of the mice sleeping against dose on a logarithmic scale, b) plotting mean induction time over a range of doses against the logarithm of the dose and c) comparison of induction times at 1.25 HD₅₀. All doses were given over 1 s or 10 s. A 1-s injection was thought to be of most value in the of structure activity effects.

Chen et al. (1966) tested the anesthetic activity and the neuropharmacological spectrum of ketamine (CI-581) in mice, pigeons and monkeys.

Child et al. (1971) tested the anesthetic activity of alfaxalone (CT1341) in mice, rats, rabbits, cats, dogs and monkeys.

Janssen et al. (1975) tested onset and duration of anesthesia after etomidate in mice, rats, guinea pigs and dogs.

New intravenous anesthetics were reviewed by Reilly and Nimmo (1987).

The anesthetic potency of remifentanyl in dogs in terms of reduction of enflurane MAC was tested by Michelsen et al. (1996).

Dingwall et al. (1993) described the tolerometer as a fast, automated method for the measurement of righting reflex latency.

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E.9.1.3

EEG Threshold Test in Rats

PURPOSE AND RATIONALE

The electroencephalographic (EEG) threshold test has been used to determine and compare the potency of several CNS depressant agents (Bolander et al. 1984; Koskela and Wahlström 1989; Norberg and Wahlström 1988; Norberg et al. 1987). Korkmaz and Wahlström (1997) described in detail the protocol of the EEG burst suppression threshold test for the determination of CNS sensitivity to intravenous anesthetics in rats.

PROCEDURE

Adult Sprague-Dawley rats are housed at a reversed light/dark cycle and an ambient temperature of $23 \pm 1^\circ\text{C}$. Twenty four hours prior to the EEG threshold test, the rats are placed in a tube restrainer. Twisted stainless steel wire and suitable surgical needles are used to sew the electrodes to the scalp above the frontal cortex. Since generalized changes in EEG recordings are used, this stainless steel material is adequate for recording purposes. Care is taken to prevent irritation of periosteal tissue. Since this procedure causes little discomfort, the use of local anesthetics and general anesthesia can be avoided.

For EEG threshold testing, the rat is placed on a warm cloth and held gently by the assistant. A needle is placed on one lateral tail vein and connected with an infusion pump. Crocodile clips are used to connect the electrodes to the EEG recorder and a crocodile clip is attached to one of the ears of the rats as a signal ground.

The EEG recording is closely observed by the technician. The changes in the EEG induced by the anesthetic agent are used to measure drug effects on the CNS. The normal EEG in an awake rat has a low amplitude and a frequency of approx. 30 cycles/s. During the first part of infusion, an increase in amplitude and a slight decrease in frequency are observed. At this stage of infusion, dependent on the anesthetic agent, jerks or sometimes convulsive episodes may occur. As the infusion continues, the frequency decreases and burst suppression periods appear. The loss of righting reflex occurs at this stage. When a burst suppression lasts one second or, the threshold criterion which is called 'silent second' is reached and the time is recorded. After the threshold determination, the rats are placed in the recovery room.

EVALUATION

The threshold dose is calculated by multiplying the time required to reach the threshold criterion with the dose administration rate. Threshold doses are determined for each anesthetic at various dose administration rates indicating the optimal dose administration rate.

MODIFICATIONS OF THE METHOD

Wauquier et al. (1988) studied relationships between quantitative EEG measures and pharmacodynamics of alfentanil in dogs. Before, during and up to 3 h after infusion, the effects of 3 doses on 6 quantitative EEG measures (zero-crossing frequency, root mean square amplitude, spectral edge, relative delta, alpha and beta power) were assessed.

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E.9.1.4

Efficacy and Safety of Intravenous Anesthetics

PURPOSE AND RATIONALE

Besides determination of the ratio between anesthetic and lethal dose, intravenous anesthetics have to be tested for their influence on the cardiovascular and pulmonary system. Borkowski et al. (1990) described a method to compare intravenous anesthetics in rabbits.

PROCEDURE

Adult New Zealand White rabbits with a mean weight of 4.5 kg are used. To provide access for direct blood pressure measurement and arterial blood samples, an 18-gauge catheter is implanted into the left carotid artery under halothane anesthesia. Following a minimum 24-h recovery period, the rabbit is placed in a sling and a pneumograph is fitted around the rabbit's caudal thorax at the level of 10th to 12th ribs to monitor respiratory rate and pattern. From the arterial catheter blood is withdrawn for blood gas analysis. Then the catheter is connected to a blood-pressure transducer. A 10-min acclimatization period is allowed before control measurements are recorded. Each rabbit serves as its own control in that cardiopulmonary parameters and responses to noxious stimuli are determined before anesthesia is induced. The right marginal ear vein is catheterized with a 22-gauge catheter, which is secured with adhesive tape, flushed with physiologic sterile saline and used for the administration of the anesthetic agents.

One third of the dose of the anesthetic to be tested is injected manually over a 1-min period. When the rabbit is relaxed it is removed from the sling and is placed in left lateral recumbence on a heating blanket. The degree of muscle tension and reaction to noxious stimuli are determined while the rabbit is in the sling and at 15 min intervals following anesthesia. The assessments performed include those of jaw tone, leg muscle tone, palpebral reflex, corneal reflex, ear pinch reflex and pedal withdrawal reflex. Jaw tone is evaluated

subjectively by pulling the lower jaw open by an index finger. Leg muscle tone is evaluated by flexion and extension of the right rear leg according to subjective scores. The corneal reflex is tested by placing a moistened cotton swab on the cornea. The palpebral reflex is tested by touching the medial canthus with a dry cotton swab. Assessment of the ear pinch reflex is performed by applying a compression force with an alligator clip. The pedal withdrawal reflex is determined by applying the same clip on the right rear fifth digit at the distal phalanx.

Cardiopulmonary parameters and rectal body temperature are determined while the rabbit is in the sling and also at 15 min intervals following induction of anesthesia with the rabbit in lateral recumbency. Heart rate, mean arterial blood pressure, respiratory rate and respiratory pattern are calculated from tracings from the physiological recorder. Arterial blood pH, partial pressure of oxygen (PaO₂), and partial pressure of carbon dioxide (PaCO₂) are determined from arterial blood samples.

EVALUATION

The heart rate, mean arterial blood pressure, respiratory rate, pH, PaO₂ and PaCO₂ are analyzed using a two-factor analysis on repeated measures. The control values are treated as covariate to allow standardization of the inherent variation between rabbits. The single *t*-test for paired differences is used to compare control values to data obtained during the later testing intervals. The standard error of the mean (SEM) is calculated for each variable at each time interval. Data for muscle tone and responses to noxious stimuli are calculated as frequency percentages. The Fisher's Exact test is used to compare between treatments. For all of the statistical analyses, a *p*-value of less than 0.05 is considered significant.

MODIFICATIONS OF THE METHOD

Details of anesthesia in the rabbit were also described by Murdock (1969).

Peeters et al. (1988) performed a comparative study of four methods for general anesthesia in rabbits.

Glenn (1980) examined the anesthetic activity of propofol (ICI 35868) in mice, rats, rabbits, cats pigs and monkeys including cardiovascular and respiratory parameters and EEG studies.

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E.9.2

Inhalation Anesthesia

E.9.2.1

General Considerations

PURPOSE AND RATIONALE

The efficacy and safety of new inhalation anesthetics has to be evaluated in pharmacological experiments. Robbins (1946) defined the anesthetic AD_{50} as the concentration of anesthetic at which 50% of mice failed to right themselves for 15 s when placed in a rotating bottle with a known concentration of anesthetic. The concentration of the anesthetic that caused apnea in 50% of the mice in 10 min was defined as the LD_{50} and the ratio LD_{50}/AD_{50} as an index of safety.

Wolfson et al. (1972) recommended brain anesthetic concentration for construction of anesthetic indices.

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E.9.2.2

Screening of Volatile Anesthetics

PURPOSE AND RATIONALE

A simple technique for preliminary testing of anesthetic agents is the introduction of a measured amount of volatile liquid into a covered glass container of known volume. If the density and molecular weight of the liquid are known, the volume % concentration of the anesthetic mixture can be calculated (assuming 1 mol of vapor = 22.4 l). Mice or rats are introduced into the chamber and the quality of anesthesia is noted. Conditions are then adjusted until the anesthetic concentration has been established.

PROCEDURE

Male NMRI mice weighing 20–25 g or male Wistar rats weighing 250–300 g are used. A wide-mouth, screw-cap glass jar of 3 Liter volume is flushed with oxygen for 1 min and a measured amount of the volatile substance is placed on the bottom through a suitable syringe. The amount is calculated to give 1.25 vol% concentration of vapor in the jar (or a logarithmic multiple of 1.25%, i. e., 0.63, 2.5, 5.0, 10.0). The jar is closed and evaporation of the substance is facilitated by gentle rotation of the jar. One rat or 5 mice are quickly placed from a beaker into the jar, and the jar is immediately closed. Every 15 s the jar is gently rotated and the time noted for each animal to become anesthetized (loss of righting reflexes). The procedure is repeated until all animals are anesthetized. Induction should occur not sooner than 30 s and not later than 5 min. The animals are allowed to remain in the anesthesia jar for 10 min, with testing of righting reflexes until they are quickly removed into room air. The time of recovery to righting or walking is recorded for each animal. Postanesthetic analgesia is tested by gently pressing the base of the tail every min until recovery has occurred. Recovery time is defined as the time in min until the mouse spontaneously moves in upright position. If induction time is shorter than 30 s or longer than 10 min, the concentration of anesthetic is decreased or increased until the proper concentrations is found.

EVALUATION

The results are reported as mean induction time and mean recovery time. Twenty-four-hour survival rate is recorded for latent toxicity.

MODIFICATIONS OF THE METHOD

Burns et al. (1961) used a simplified mouse test apparatus with a small container and an open circuit technique.

Raventós (1956) used cats, dogs and monkeys to evaluate the cardiovascular effects of fluothane.

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E.9.2.3

Determination of Minimal Alveolar Anesthetic Concentration (MAC)

PURPOSE AND RATIONALE

The term “**minimum alveolar anesthetic concentration**” (**MAC**) was coined by Merkel and Eger (1963) as an index to compare various anesthetic agents.

The use of *MAC* which represents the partial anesthetic pressure in the brain has gained wide acceptance (Eger et al. 1965; Quasha et al. 1980).

For **man**, Saidman and Eger (1964) defined *MAC* as the point at which 50% of the patients moved in response to a surgical incision.

A method for determining minimum alveolar concentration of anesthetic in the **rat** was published by Waizer et al. (1973). Kashimoto et al. (1997) determined the minimum alveolar concentration of sevoflurane in rats. Eger et al. (1999) studied maximum alveolar anesthetic concentration of fluorinated alkanols in rats and discussed the relevance to theories of narcosis. Eger et al. (2003) studied additive minimum alveolar concentration (*MAC*) effects of halothane and isofluroane in rats.

Issues in the design and interpretation of minimum alveolar anesthetic concentration (*MAC*) studies were discussed by Sonner (2002).

PROCEDURE

Minimum alveolar anesthetic concentrations (*MAC*) are determined in Sprague Dawley rats weighing 300–450 g. Each rat is placed in an individual gas tight plastic cylinder closed at both ends by rubber stoppers. The stoppers are pierced with holes for various purposes. A rectal temperature probe (temperature maintained between 36°C and 38.5°C) and the rat’s tail are drawn separately through holes in the rubber stopper closing the distal end of the cylinder. Delivered gases at an average inflow rate of 1L/min to each rat enter through ports at the head (proximal) end of the cylinder and exit at the tail (distal end), a flow to minimize rebreathing (inspired CO₂ < 10 mm Hg). Exiting gases are scavenged.

The anesthetics are introduced from conventional vaporizers. For the determination of *MAC*, an initial concentration is used that permits movement of the rats

in response to noxious stimulation. A tail clamp is applied for 1 minor until the animal moves, and the anesthetic partial pressure is measured by gas chromatography. If the animal moves, the partial pressure is increased by 0.2% or 0.3% atmospheres. After equilibration for 30 min, the tail clamp is applied again and the anesthetic partial pressure measured by gas chromatography. This procedure is repeated until the partial pressures bracketing movement-nonmovement are determined for each rat.

EVALUATION

MAC is defined as the average of the partial pressures that just prevented movement in response to clamping of the tail. Differences between anesthetics are accepted at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Fang et al. (1997) found that maturation decreases ethanol minimum alveolar anesthetic concentration (*MAC*) more than desflurane *MAC* in **rats**.

Gong et al. (1998) assessed the effect of rat strain on susceptibility to anesthesia and convulsions produced by inhaled compounds in five different rat strains. Strain minimally influenced anesthetic and convulsant requirements of inhaled compounds in rats.

Doquier et al. (2003) studied the minimum alveolar anesthetic concentration of volatile anesthetics in rats as tools to assess antinociception in animals.

Determination of the minimal alveolar concentration (*MAC*) of halothane in the white New Zealand **rabbit** was published by Davis et al. (1975).

Determination of an anesthetic index (*Apnea/MAC*) in experiments in **dogs** has been proposed by Regan and Eger (1967).

Murphy and Hug (1982), Hall et al. (1987) used the reduction of enflurane *MAC* values in **dogs** as parameter for the anesthetic potency of fentanyl or sufentanyl, respectively.

Seifen et al. (1987) used *MAC* values for comparison of cardiac effects of enflurane, isoflurane, and halothane in the **dog heart-lung preparation**.

Ide et al. (1998) used airway occlusion in **cats** as a noxious respiratory stimulus that induces a visceral sensation of choking for determination of minimum alveolar anesthetic concentrations during halothane, isoflurane, and sevoflurane anesthesia. These values were compared with *MAC* values for somatic noxious stimuli such as toe pinch or tetanic stimulus. The authors recommended this method as a new concept for *MAC* determination.

Eger et al. (1988) determined minimum alveolar concentration of fluorinated anesthetics in **pigs**.

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E.9.2.4

Efficacy and Safety of Inhalation Anesthetics

PURPOSE AND RATIONALE

To assess the safety margin of a inhalation anesthetic not only the *ED*₅₀ values but also the maximally effective dose and the dose with a minimal danger of fatal outcome should be determined. In particular, *cardiovascular parameters* are observed (Kissin et al. 1983).

PROCEDURE

Male Sprague Dawley rats weighing 300–350 g are placed into a clear chamber with the tail protruding from a special opening. An anesthetic-oxygen non-humidified mixture is directed into the chamber at a rate of 4 l/min. The inhalation anesthetics, e. g., halothane or isoflurane, are vaporized in Draeger vaporizers and the level in the chamber is monitored with a gas analyzer which is calibrated with a mass spectrometer. Rectal temperature is monitored and maintained at 37°C with a heating pad. Each rat is exposed to only one pre-determined concentration of anesthetic for 30 min, at which time the presence or absence of the end point of anesthesia is determined. For the lethal end point, rats are tracheotomized and ventilated at 60 strokes/min through an endotracheal catheter. Tidal volume is adjusted to maintain PaCO₂ at 40 ± 5 mm Hg.

As endpoints of anesthesia are used:

- Loss of righting reflex. The test is regarded as positive if the animal fails to right itself with all four feet on the floor within 15 s after being placed in a side position.
- Prevention of purposeful movements response to a noxious stimulus. The animals are stimulated for 60 s by placement of a 1-kg weight on the middle of the tail. Only the purposeful movement of the head or legs is considered to be a response.
- Prevention of the heart rate increase to a noxious stimulus (ECG signals). An increase in heart rate of greater than 1% is regarded as a positive response.
- The end point for the lethal effect is 7 mm Hg in the femoral artery with artificial respiration.

With each of the anesthetics, four series of experiments are performed: to determine the righting reflex, purposeful movement response, heart rate response,

and lethal effect. The concentrations of the test compounds and the standard are spaced equally between the above-mentioned doses.

After determination of the heart rate effect and the lethal effect, the rats are sacrificed for determinations of brain tissue concentrations. The whole brain is removed and tissue anesthetic concentration is determined by gas chromatography.

EVALUATION

For calculation of the dose-effect curves, the probit method of statistical analysis is used.

For the assessment of anesthetic safety, not only the therapeutic ratio (LD_{50}/ED_{50}) but also the standard safety margin

$$SSM = (LD_5 - ED_{95})/ED_{95} \times 100$$

is used. This represents the percentage by which the ED_{95} has to be increased before LD_5 is reached.

CRITICAL ASSESSMENT OF THE METHOD

The standard safety margin has definitive advantages over therapeutic ratio. In contrast to the LD_{50}/ED_{50} index, the standard safety margin is influenced not only by the distance between central points of the anesthetic and lethal dose-effect curves, but also by the slope of these curves.

MODIFICATIONS OF THE METHOD

A similar concept, based on response to tail clamping, respiratory arrest and cardiovascular failure in the **rat** was published as anesthetic index by Wolfson et al. (1973).

An other attempt to determine anesthetic requirements in rats was published by White et al. (1974).

Kissin et al. (1984) studied the morphine-halothane interaction in rats.

Fukuda et al. (1996) investigated the effects of sevoflurane and isoflurane on bupivacaine-induced arrhythmias and seizures in rats.

Kanaya et al. (1998) Compared myocardial depression by sevoflurane, isoflurane, or halothane in **cultured neonatal rat ventricular myocytes**. Changes in beating rate and amplitude during exposure to the anesthetics were measured.

Chaves et al. (2003) used non-invasive electrocardiography in **mice** to study the effects of intravenous and inhalation anesthetics and of age.

Krantz et al. (1941, 1953) described an anesthetic index between surgical anesthesia (cornea and wink reflexes abolished) and respiratory failure in **dogs**.

Van Poznak and Artusio (1960a, b) determined the anesthetic properties of fluorinated compounds in dogs using a face mask for the induction of anesthesia and a cuffed endotracheal tube later on. ECG (lead II) and EEG were monitored.

Steffey and Howland (1978) determined the potency of enflurane in dogs in comparison with halothane and isoflurane.

Johnson et al. (1998) compared isoflurane with sevoflurane for anesthesia induction and recovery in adult dogs.

Salmempera et al. (1992) studied in dogs the potency of remifentanyl, a short acting opioid analgesic, which is used as anesthetic adjunct by variable-rate infusion. Enflurane minimal alveolar concentration was measured by the tail-clamp method in dogs before and after sequential infusion of various doses of remifentanyl. The plasma concentration causing a 50% reduction of enflurane minimal alveolar concentration was determined.

Kataoka et al. (1994) studied the negative inotropic effects of sevoflurane, isoflurane, enflurane and halothane in canine blood-perfused papillary muscles.

Hirano et al. (1995) compared the coronary hemodynamics during isoflurane and sevoflurane anesthesia in dogs.

Mutoh et al. (1997) compared the cardiopulmonary effects of sevoflurane with those of halothane, enflurane, and isoflurane, in dogs.

Hashimoto et al. (1994) examined the effects of sevoflurane and halothane on the effective refractory period and ventricular activation in a canine myocardial infarction model.

The effects of desflurane, sevoflurane and halothane on postinfarction spontaneous dysrhythmias in dogs were examined by Novalija et al. (1998).

Cardiopulmonary effects in **cats** were studied for desflurane by McMurphy and Hodgson (1996), for sevoflurane by Hisaka et al. (1997).

Saeki et al. (1996) determined the effects of sevoflurane, enflurane, and isoflurane on baroreceptor-sympathetic reflex in **rabbits**.

Hanagata et al. (1995) found that isoflurane and sevoflurane produce a dose-dependent reduction in the shivering threshold in rabbits.

Antognini and Eisele (1993) determined anesthetic potency and cardiopulmonary effects of enflurane, halothane, and isoflurane in **goats**.

The effects of multiple administrations of sevoflurane to cynomolgus **monkeys** were evaluated by Soma et al. (1995).

The effect of inhalation anesthetics on the **respiratory system** was investigated in several studies:

Mazzeo et al. (1996) compared the relaxing effects of desflurane and halothane at various MACs on isolated proximal and distal airways of dogs precontracted with acetylcholine.

Hashimoto et al. (1996) compared the bronchodilating effect of sevoflurane, enflurane and halothane in dogs using a superfine fiberoptic bronchoscope. The dogs were anesthetized with pentobarbital, paralyzed with pancuronium, and the lungs were mechanically ventilated. The endotracheal tube had an additional lumen to insert the superfine fiberoptic bronchoscope (outer diameter 2.2 mm) which was located between a second and third bronchial bifurcation to continuously monitor the bronchial cross-sectional area of third or fourth generation bronchi. Bronchoconstriction was produced by histamine injection and infusion. The bronchial cross-sectional area was printed out by a video-printer at the end of expiration and was calculated on a computer using an image program after various MACs of the different inhalation anesthetics.

Mitsuhashi et al. (1994) induced systemic anaphylaxis in dogs sensitized to *Ascaris suum* by intravenous injection of the antigen and measured pulmonary resistance and dynamic pulmonary compliance. Sevoflurane was as effective as isoflurane in attenuating bronchoconstriction associated with anaphylaxis in dogs.

Cervin and Lindberg (1998) examined the short-term effects of halothane, isoflurane and desflurane on mucociliary activity in the rabbit maxillary sinus *in vivo*.

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E.10 NMRI Methods in Psychoneuropharmacology

E.10.1 General Considerations

PURPOSE AND RATIONALE

Magnetic resonance imaging (MRI) is the preferred technique for the visualization of lesions in the brain and spinal cord of patients with MS. It visualizes the resonance signals of tissue protons when they are placed in a time-varying strong magnetic field. The most frequently used parameters measured in MS are the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1-mm-thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T_2 images are sensitive to water and, because all pathological alterations in MS brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualization of the spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T_1 values, whereas relatively loose structures, such as gray matter or lesions, have higher T_1 values.

To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected (0.1–0.3 mmol/kg) and, in areas of increased blood–brain barrier permeability, leaks into the brain parenchyma, causing local enhancement of the T_1 -weighted signal intensity.

A third important MRI technique in MS is magnetization transfer ratio (MTR) imaging. The MTR of a given tissue is defined as the ratio of free protons versus protons bound to tissue macromolecules.

Besides general applications to body composition in animals (Mitchell et al. 2001; Changani et al. 2003;

Mirsattari et al. 2005) NMR studies contributed to evaluation of drugs in psychoneuropharmacology.

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E.10.2 NMRI Psychopharmacological Studies in Rats

E.10.2.1 NMRI Study of Experimental Allergic Encephalomyelitis in Rats

PURPOSE AND RATIONALE

Duckers et al. (1997) studied the effect of a neurotropic treatment on cortical lesion development in experimental allergic encephalomyelitis in rats by longitudinal *in vivo* magnetic resonance imaging methods.

PROCEDURE

Animals

Female Lewis rats weighing approximately 190 g were used for *in vivo* proton MRI. The rats were housed individually in Macrolon cages and had free access to rat chow and water during the 5-month study. The rats were divided at random into three groups. In two groups of rats chronic experimental allergic encephalomyelitis was induced by s.c. injection of myelin/adjuvant. The third group was injected with vehicle.

Immunization of Animals

Two hours before inoculation, spinal cords from Duncan Hartley guinea pigs were dissected and homogenized in an equal volume of phosphate-buffered saline (PBS) (pH 7.3; 1 g spinal cord in 1 ml PBS). Equal parts of guinea pig spinal cord homogenate in PBS and complete Freund's adjuvant (CFA) were mixed as described by Lassmann (1983) and Wisniewski and Keith (1977).

Anesthetized rats were sensitized by injecting 0.3 ml of the CNS emulsion in the dorsum pedis of the hindlimbs. Age-matched controls were challenged

similarly except that the emulsion solely contained PBS in CFA. The day of inoculation was designated day 0 post-inoculation (dpi 0).

Peptide Treatment

For the neurotrophic treatment, an ACTH₄₋₉ analog devoid of corticotrophic activity [Org2766, H-Met(O₂)-Glu-His-Phe-D-Lys-Phe-OH] was used. The heptapeptide has well-documented neurotrophic properties, as demonstrated previously in different animal models for peripheral and central nerve damage (Bär et al. 1990; Stand et al. 1991). The rats with chronic experimental allergic encephalomyelitis were given s.c. injections, in the neck, of either the ACTH₄₋₉ analog (75 µg/kg in 0.5 ml saline) or 0.5 ml saline every 48 h. Age-matched control animals received saline injections every 48 h. Treatment was started on the day of inoculation and continued until the experiment ended 21 weeks after inoculation.

Neurological Status of the Rats

The clinical status of the rats was evaluated every 48 h by weighing the rats and scoring their neurological status on a 0–9 ordinal scale. The scores represent the following neurological symptoms: grade 0, no visible neurological symptoms; grade 1, loss of tail tip reflex; grade 2, flaccid tail; grade 3, moderate paraparesis, manifested as minor locomotion disturbances; grade 4, severe paraparesis accompanied by lordosis, severe disturbed locomotion and severe weight loss; grade 5, one paralysed hindlimb; grade 6, complete paralysis; grade 7, paralysis from diaphragm upwards; grade 8, tetraplegia, only head movements possible; grade 9, death due to chronic experimental allergic encephalomyelitis.

T₂-Weighted and Short Tau Inversion Recovery Magnetic Resonance Imaging

Animals were examined by using MRI at 5, 10, and 20 weeks post-inoculation. The rats were anesthetized with Hypnorm (containing 10 mg/ml fluanisone and 0.315 mg/ml fentanyl citrate, dose 0.6 ml/kg s.c.) and Stesolid (diazepam). After intubation, the rats were mechanically ventilated with 70% oxygen and 30% N₂O with 0.5% v/v halothane.

The ¹H-MRI experiments were performed with a SISCO 200/400 system (SISCO, Palo Alto, Calif., USA) operating at 4.7 T and equipped with actively shielded gradients (max. gradient 18 Mt/m). In all experiments, an Alderman-Grant type radio frequency coil with an inner diameter of 5 cm was used. For accurate and reproducible positioning, animals were

mounted in a stereotaxic holder positioned on the coil support. For each animal, a sagittal T1W scout image [echo time (TE) 20 ms, repetition time (TR) 600 ms] outlined the start (border of frontal lobe and olfactory bulb) and endpoint (border of cerebellum–spinal cord) for subsequent coronal multislice experiments used to quantify the extent of tissue damage.

All MRI experiments were based on a spin-echo sequence with 5-ms sinc-modulated radiofrequency pulses. A total of 11 T2W coronal multislice images per animal was obtained (slice thickness 1.1 mm, interslice gap 0.7 mm), using a TR of 2 s and a TE of 60 ms; two transients were averaged. The field of view was 5 × 5 cm²; 128 points were acquired in the phase encoding direction and 512 points in the read-out direction. After processing, these were presented in a square matrix of 512 × 512 pixels.

Subsequently, multislice coronal short tau inversion recovery (STIR) images were recorded as described above (TR/TE 2000/60, number of transients 2), except that the spin echo sequence was preceded by a 180° inversion pulse followed by an inversion time of 250 ms. In pilot experiments, this inversion time was found to yield optimal contrast between compromised and healthy brain tissue.

In order to measure the coronal relaxation time T₂ on a pixel-by-pixel basis, six rats (two age-matched control rats and four rats with chronic experimental allergic encephalomyelitis) were used in a series of T2W imaging experiments with a TE of 20 ms, 40 ms, 60 ms, and 80 ms and a TR of 6 s. In this experiment, which was performed at week 21 post-inoculation, all other parameters were identical to those of the above-mentioned T2W imaging experiment. The series of T2W images at a given slice position were used to reconstruct a two-dimensional map of T₂ by fitting the data on a pixel-by-pixel basis to monoexponential decay functions. T₂ values of cortex tissue, thalamus and ventricles were read from the T₂ map and averaged among animals. T₂ values of lesioned tissue were calculated for lesions which extended throughout the thickness of the MRI slice.

All MRI data were analysed using MRI specific software (Image Browser, SISCO, Sunnyvale, Calif., USA) by two different investigators who were blind to the treatment given to the animals. A filter was positioned over the region of interest (cortex and subcortical regions) in MRI slices. The filters were preset at fixed values, which appeared to yield an optimal differentiation between areas of relatively low and high signal intensity in pilot experiments. Pixels with relatively high signal intensities (e. g., higher than the fil-

ter setting) were added to obtain the total surface area of the brain regions with high signal intensities in that given slice. These surface areas with high signal intensity were summed for all slices for each animal and converted to total lesion volume (in μl). In the analysis, interslice gaps were not included in the calculation of the total lesion volume.

EVALUATION

Application of the drugs, evaluation of the clinical status and the MRI were all performed by different investigators who were blind to the treatment given to the animals. Treatment codes were broken at the end of the experiment after data analysis had been completed. The distribution of clinical scores in the different treatment groups was analyzed using a non-parametric Wilcoxon Rank Sum test. Treatment effects on lesion volume measured by MRI were tested by an analysis of variance for repeated measurements (MANOVA) followed by additional Student's *t*-tests (two-paired). $P < 0.05$ was considered to be significantly different.

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Krebs' cycle and mitochondrial electron transport. 3-Nitropropionic-acid-intoxicated rats serve as the animal model for one neurodegenerative disease, Huntington's disease. Chyi and Chang (1999) applied non-invasive diffusion- and T_2 -weighted magnetic resonance imaging to study the temporal evolution and spatial distribution of brain lesions which were produced by intravenous injection of 3-nitropropionic acid in rats.

PROCEDURE

Animal Preparation

Male Sprague Dawley rats were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg). Anesthesia was maintained by serial supplement of pentobarbital (5 mg/50 min, i.v.) 2 h after the initial injection. One femoral vein was cannulated for chemical administration, and an endotracheal tube was set for artificial ventilation with an animal ventilator. A muscle relaxant, gallamine was injected intravenously to avoid spontaneous ventilation and movement during the image-acquisition period. The initial dose of gallamine was 12 mg and the maintaining dose was 6 mg/h. A bolus injection of 3-nitropropionic acid was intravenously administered.

Image Acquisition

Experiments were performed on a 4.7 T spectrometer (Spectrospin, Biospec 4.7 T, Fällanden, Switzerland) with an active shielding gradient at 5.6 G/cm in 500 μs . A 70-mm birdcage RF coil was used, and rats were placed in a prone position with a custom-designed head-holder. Four transverse images (2-mm-thick) along anterior–posterior direction were obtained using a 5-cm field of view and 128×256 matrix. The offsets were set corresponding to the sections -4.3 , -1.8 , $+0.7$, and $+3.2$ mm to the bregma. A fast spin-echo sequence was applied for T_2 -weighted images (T_2 WIs) with relaxation TIME = 4000 ms, effective echo TIME = 80 ms, and echo train LENGTH = 8. For diffusion-weighted images (DWIs), the pulsed field gradient method was employed with a relaxation time of 2000 ms, echo time of 59 ms, δ of 20 ms, Δ of 27 ms, and b-value of 1300 s/mm^2 . The diffusion-sensitive gradients were applied in the read (*x*) direction before and after the 180° pulse. The controlled T_2 WIs and DWIs were acquired prior to 3-nitropropionic administration. Then 2.5 min after the injection of 3-nitropropionic, sequential T_2 WIs and DWIs were continuously performed for 8 h.

In a separate preparation, DSC MRI was performed immediately after diffusion-weighted MRI at 5 min be-

E.10.2.2

NMRI Study of 3-Nitropropionic Acid-Induced Neurodegeneration in Rats

PURPOSE AND RATIONALE

Effective clinical therapies for neurodegenerative diseases are currently lacking because the precise mechanisms underlying cell death are still obscure. It is hypothesized that a defect in mitochondrial energy metabolism may be the basis of secondary excitotoxic neuronal death in neurodegenerative diseases. 3-Nitropropionic acid is an irreversible succinate dehydrogenase inhibitor which blocks both the

fore and 4 h after 3-nitropropionic acid administration. A 2-mm-thick transverse single-slice was chosen and centered at 0.5 mm anterior to the bregma where the striatal lesion sites were easily observed on the DWIs. A series of 30 gradient-echo images with a 64×256 matrix size, relaxation TIME = 30 ms, echo TIME = 10 ms, and $\alpha = 15^\circ$ were acquired. The acquiring time for each gradient echo image was 1.92 s. The bolus of susceptibility contrast agent, gadopentonic acid (0.3 mmol/kg, Schering, Berlin, Germany), was injected intravenously 10 s after the start of image acquisition.

EVALUATION

Data processing was performed using a commercially available image analysis software (MRVision, MRVision, Menlo Park, Calif., USA). According to Paxinos and Watson (1986), selected regions of interest (ROIs) in the striatum, hippocampus, cortex, and corpus callosum were defined. A signal-to-noise ratio (SNR) of a ROI was defined as the ratio of the mean signal intensity (SI) in this ROI to the mean SI in an adjacent background area. The relative contrast of a ROI was calculated according to the following formula: $|SI_A - SI_C| / SI_C$, where SI_A is the mean SI in this ROI and SI_C is the mean SI in the cortical ROI on the same image (Knowles and Markisz 1988). The SI_C was selected as reference because its SNR remained constant throughout the experimental period. The rCBV maps were generated by the integral under the ΔR_2^* transit curves on a pixel-by-pixel basis. The SNR, relative contrast, and regional rCBV determined from the rCBV map were all unit-less ratios.

Results were presented as means \pm SEM. Statistical analyses were performed using paired Student's *t*-test to compare controlled and 3-nitropropionic-acid-treated data. $P < 0.01$ was considered statistically significant.

MODIFICATIONS OF THE METHOD

Using magnetic resonance imaging and *in vivo* proton magnetic resonance spectroscopy, Lee et al. (2000) evaluated the neuroprotective effect of lamotrigine and MK-801 on rat brain lesions induced by 3-nitropropionic acid.

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E.10.2.3

NMRI Studies of Brain Activation in Rats

PURPOSE AND RATIONALE

Brain functional magnetic resonance imaging (fMRI) is capable of revealing *in vivo* biochemical, structural, and functional information that reflects the physiological integrity of the brain (Jenkins et al. 1996). In general, brain fMRI includes four different aspects of MR methods including tissue water movement (diffusion-weighted MRI) (Le Bihan et al. 1986), capillary flow (perfusion-weighted MRI) (Williams et al. 1992; Kim 1995), neuronal activation (blood oxygenation level-dependent, BOLD, MRI) (Ogawa et al. 1992), and cerebral metabolites (magnetic resonance spectroscopy) (Brown et al. 1982). In all of these methods, the BOLD effect, based on the difference in magnetic susceptibility between paramagnetic blood deoxyhemoglobin and surrounding tissues, is frequently employed to *in vivo* map functional changes during brain activation (McCarthy et al. 1993; Turner et al. 1993).

Chang and Shyu (2001) described a functional magnetic resonance imaging (fMRI) study of brain activations during non-noxious and noxious electrical stimulation of the sciatic nerve of rats.

PROCEDURE

Male Sprague Dawley rats were initially treated with atropine (6 mg/kg, i.p.), and then anesthetized with methohexital sodium, 65 mg/kg, i.p. A PE-240 tube was inserted via tracheotomy for artificial ventilation. A PE-50 catheter was placed in the femoral vein for the administration of drug. α -Chloralose (50 mg/kg) was given intravenously. After the sciatic nerve was exposed and isolated, a cuff electrode made from silicon tube and stainless steel wires was placed around the nerve. The surrounding muscle and skin were carefully sutured, and paraffin oil was filled around the electrode to prevent short-circuiting. Artificial ventilation was applied and CO_2 concentration was monitored and controlled within the range of 3.5%–4.5%. Rectal temperature was measured and maintained at about $37^\circ C$ by a feedback-controlled warm-air system. After the

head-holder was in place, muscle relaxant (gallamine triethiodide, 50 mg/kg, i.v.) was given for muscle relaxation.

Electrophysiological Experiment

Laminectomy was performed between T12 and L5. Dorsal roots at L4 and L5 level were carefully isolated and cut. Each of the peripheral ends of the roots was ligated with a cotton thread previously soaked with normal saline. A silver-silver chloride electrode hooked on the end of the root was used to record the evoked field potentials extracellularly ipsilateral to the stimulating sites. The extracellular field potentials were amplified by a high-input impedance amplifier. All analog signals were displayed on either an analog or digital storage oscilloscope. Twenty sweeps of evoked compound potentials were averaged for each stimulus intensity. The threshold current for inducing minimal muscle twitch was tested before applying the muscle relaxant. The electrical current was delivered by a constant current pulse generator. The electrical pulse was a square wave, 0.5 ms duration, 2 Hz. This muscle twitch threshold current was used to compare with the threshold for inducing the minimal compound action potentials. The intensity was increased as a multiple of the threshold current value.

Imaging Acquisition in fMRI Experiments

MR experiments were performed on a Biospec BMT 47/40 4.7 T system equipped with an actively shielded gradient system (up to 5.9 G/cm in 500 μ s). A 70-mm birdcage RF coil was used, and rats were placed in a prone position with a custom-designed head-holder. T1-weighted spin-echo images were scanned in transverse and sagittal planes to position the animal properly so that images were centered 1 mm posterior to the bregma. For identifying anatomical location, T2-weighted spin-echo images with four slices in the transverse plane were acquired using a repetition time (TR) of 4 s, an echo time (TE) of 80 ms, a field of view (FOV) of 5 cm, and a slice thickness (SLTH) of 2 mm. The acquisition matrix was 256 \times 128 with a matrix of 256 \times 256 after zero-filling. Thus, the images covered the rat brain from 3 mm anterior to the bregma to 5 mm posterior to the bregma. At the same locations of T2-weighted spin-echo images, a series of 40 gradient-echo images per slice for four slices in the transverse plane were collected for baseline and stimulation sets with a TR of 180 ms, an TE of 20 ms, a flip angle of 22.5°, a FOV of 5 cm and a SLTH of 2 mm. The acquisition matrix was 256 \times 64 with a matrix of 256 \times 256 after zero-filling. The total scanning time for

each image was 23 s. In the baseline set, the stimulation was turned off during the first eight image acquisitions. The electrical stimulation was switched on in two stimulation sets during acquisition of images 9–16 and 25–32. The stimulator was switched off for the rest of the period. The stimulation consisted of rectangular pulses of 0.5 ms duration and a 2 Hz frequency current. The same muscle twitch measurement as described in the electrophysiological study session was conducted to determine the intensity of the threshold current. In each image acquisition session, only one stimulation intensity was used. Stimuli with varied threshold-multiplied intensities were applied randomly in three different sessions in each rat. At least 30 min waiting time was allowed between each acquisition session.

Physiological Data Acquisition System in fMRI Experiments

Inside the magnet, animals were placed in an acrylic head-holder for accurate positioning of the location of brain structures. The integrated physiological data acquisition system we used was processed and controlled by a program written in Microsoft BASIC language on an IBM personal computer (Shyu et al. 1996). The data acquisition and control signals were channeled through serial ports and a PCL-818 (Advantech, A/D converter and digital input/output) interface card. All digitized physiological data, timing events and trigger pulses from each image acquisition were displayed in real time to view the animal's condition. The same data were placed in a data array and stored on a hard disk for off-line analyses, plotting, and printing.

EVALUATION

Image data processing and analyses of significance of signal changes were performed using MRVision (MRVision, USA) running on a Silicon Graphics INDY workstation. The series of multi-sliced images were realigned in the vertical and horizontal direction related to the first image in the series. The analyses of the aligned data were based on the cross-correlation processing strategy (Bandettini et al. 1993). The correlation coefficient between each pixel of the series of images and a model of a square wave (whose OFF-ON-OFF-ON-OFF period was the same as the stimulation pattern) was calculated. An associated *t*-statistic image was generated by the fitting function. The *t*-statistic image was used to determine whether the correlation coefficient for each pixel was significantly different from zero. The same analysis procedure was carried out in each slice of the four slices. A critical *t*-value for each pixel for the desired level

of significance (e. g., $P < 0.01$) and degrees of freedom (total images-2) was calculated and an output image, an activated map consisting of clusters of pixels whose values exceeded the level of significance, was generated. This image, white color in appearance, was then overlaid with a high-resolution T₂-weighted image, resulting in an image showing the significantly activated region in white overlapping the anatomical image. The region of interest (ROI) from the *t*-statistic image was selected according to its corresponding location in the T₂-weighted image. The stereotaxic atlas of the rat (Paxinos and Watson 1998) was used as the reference for determining the corresponding anatomical structures in T₂-weighted images. The time course of signal intensities of 40 images of each of the four slices was obtained in the selected ROI. The extent of the functional activation was obtained by calculating the percentage change of averaged signals between the OFF period and the first and the second ON periods. Each chosen ROI corresponded to an anatomical structure that appeared in different slices, thus the signal intensity changes of each ROI in different slices could be averaged. The area of each ROI as depicted by pixel numbers appearing in different slices was summed. The mean percent signal increases and total area of each ROI activated by different intensities were compared using one-way ANOVA with $P < 0.05$ or < 0.001 considered to be significant. Post-hoc examination of the difference between groups was performed by the Student–Newman–Keuls test.

MODIFICATIONS OF THE METHOD

Kerskens et al. (1996) compared ultrafast perfusion-weighted MRI of functional brain activation in rats during forepaw stimulation with T₂*-weighted MRI.

Several other studies using MRI were performed in rats:

Wrynn et al. (2000) reported an *in vivo* magnetic resonance imaging study of the olfactory bulbectomized rat model of depression. The magnetic resonance imaging (MRI) investigation demonstrated alterations in signal intensities in cortical, hippocampal, caudate and amygdaloid regions in olfactory bulbectomized animals, but not in sham-operated controls. Ventricular enlargement was also evident in olfactory bulbectomized animals.

Itoh et al. (2004) described magnetic resonance and biochemical studies during pentylentetrazole-kindling development and the relationship between nitric oxide, neuronal nitric oxide synthase and seizures. The daily administration of pentylentetrazole was associated with an increase in the amount of neuronal ni-

tric oxide synthase (nNOS). NO generation was measured directly by *in vivo* and *ex vivo* electron paramagnetic resonance on rats undergoing progressive convulsions. Morphological changes in the brain structure of rats were measured by magnetic resonance imaging during epileptic convulsions induced by repetitive administration of pentylentetrazole.

Watanabe et al. (2006) described mapping of the habenulo-interpeduncular pathway in living mice using manganese-enhanced 3D MRI. Six hours after intracerebroventricular microinjection of MnCl₂, T1-weighted 3D MRI (2.35 T) at 117 μm isotropic resolution revealed a continuous pattern of anterograde labeling from the habenula via the fasciculus retroflexus to the interpeduncular nucleus.

Hasegawa et al. (2003) described diffusion-weighted imaging in kainic-acid-induced complex partial status epilepticus in dogs. A cannula was stereotactically inserted into the left amygdala. One week after surgery, all dogs were imaged using MRI. Pre-injection imaging consisted of T2-weighted (T2W) imaging, fluid attenuated inversion recovery (FLAIR), and DWI. Two weeks after surgery, five dogs received intra-amygdaloid KA microinjections. MRI was carried out at 3, 6, 12, 24, and 48 h after onset of complex partial status epilepticus.

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E.10.3

NMRI Psychoneuropharmacological Studies in Primates

E.10.3.1

Functional NMRI Studies in the Brain of Rhesus Monkeys

PURPOSE AND RATIONALE

Zhang et al. (2000) and Andersen et al. (2002) published methodological details for functional MRI studies in awake rhesus monkeys.

PROCEDURE

Adult female rhesus monkeys (*Macaca mulatta*) were maintained on a 12-h light:12-h dark cycle in individual cages with water available ad libitum. They had to be adapted to an MRI environment.

The goal was to elicit cooperative involvement of the rhesus monkeys through positive reinforcement. The animals were first acclimated to working with the trainers in their home cage room. Visual contact, vocal familiarity and positive reinforcements (juice rewards and preferred foods) were used to promote bonding

and establish a working relationship between subject and trainer. A pole-and-collar system was used to handle and train monkeys. The monkeys were trained to voluntarily exit their home cages with use of positive reinforcements and the pole-and-collar system. After subjects were sufficiently trained, they were introduced to the primate-training chair. Food was removed from the subject's home cage approximately 20 h prior to a training session. Training sessions lasted 45–75 min and were conducted at least twice a week per subject until no major movements were observed.

Apparatus

The animals were adapted to the MRI-compatible chair constructed from non-ferromagnetic materials and designed to comfortably position an adult rhesus monkey in a prone, sphinx-like position within the magnet bore. The head-holder was designed to restrict head motion without having to surgically attach a head-holder to the skull. The head frame was placed over the monkey's head and secured to the training chair using four nylon screws. The monkey's head rested comfortably on a padded chin support that acted as a stabilizer and cushion. Its nose and mouth were positioned outside the head-holder for comfort and ease of breathing. Under local anesthesia (1% lidocaine, 2.0 ml on each side), two MRI-compatible pins were inserted through the overlying skin and connective tissue to contact the bony cranium. Earbars/earplugs were constructed that followed the natural angle of the rhesus ear canal and secured firmly to the chair-mounted head-holder. Earbars/earplugs were used both for MRI and training sessions to reduce head movement and protect the animals from the high ambient MRI noise levels. Two separate boxes in front of the MRI-compatible chair accommodated the front limbs of the monkey.

fMRI Procedures

The scans were conducted on a Siemens VISION (VB33A) 1.5T MRI scanner using the body coil to transmit radio frequency and an 8-cm surface coil placed above the monkey's head for RF signal reception. The anatomic structures of interest were visualized using a 3D FLASH sequence with 1 mm isotropic resolution (TR/TE = 21/6 ms, flip ANGLE = 30°, image matrix SIZE = 128 × 128 × 90, field of VIEW = 128 mm, BANDWIDTH = 195 Hz per pixel). No correction was made for the fall-off in signal intensity with distance from the surface coil in the anatomic images, but sufficient sensitivity was

available to accurately visualize anatomic structures throughout the entire slice.

Functional MR images were acquired continuously using a FLASH 2D multiple gradient-recalled-echo (MGRE) navigator sequence. Slices were acquired interleaved for three non-contiguous positions in the coronal plane: the first slice covering the area of the putamen and the caudate, the second one covering globus pallidus, and the last one included the substantia nigra (Zhang et al. 2000). An 11-echo MGRE sequence was used to map on a pixel-by-pixel basis the local transverse relaxation rate R_2^* (Menon; Andersen; Chen and Chen). R_2^* is the local transverse relaxation rate for the measured MR signal intensity, $S(\text{TE}) = S_0 \exp(-R_2^* \text{TE})$, where TE is the echo delay time. It is the parameter that affects contrast in a T_2^* -weighted image sensitized to local susceptibility effects such as those caused by changes in the blood oxygenation level. Darker regions in T_2^* -weighted images have larger baseline R_2^* values reflecting a more rapid MR signal decay rate ($R_2^* = 1/T_2^*$). The increase in blood oxygenation level associated with brain "activation" causes a decrease in the value of the R_2^* parameter. There are several advantages of using R_2^* . As an intrinsic MR parameter, its value is not affected by the non-uniform sensitivity pattern of the surface coil used for signal reception, provided the images at all echo times TE have sufficient signal level, well above the intrinsic noise floor. Also, R_2^* is insensitive to changes in the absolute image intensity, S_0 , that may occur over the duration of a long scan. A disadvantage is that the rate of acquisition is slower, although a single-shot multi-echo EPI method was recently developed for quantitative R_2^* mapping in functional MR imaging (Posse et al. 1999).

The last TE images were acquired without phase encoding gradients to serve as a navigator echo for detection of head motion occurring during the acquisition of data for reconstruction of single R_2^* images. The FLASH 2D acquisition parameters were: TR = 250 ms, TE = 7–75 ms, image acquisition matrix SIZE = 112×128 , rectangular field of VIEW = 112×128 mm, slice THICKNESS = 3 mm, flip ANGLE = 40° , BANDWIDTH = 156 Hz per pixel. Local shimming of the static magnetic field B_0 in the basal ganglia was used to optimize sensitivity to the BOLD effect. For each animal, 20 min of baseline fMRI data were collected, after which 0.1 mg/kg apomorphine was injected subcutaneously. Data collection then resumed for another 20 min. In cases where replicate scans were obtained in the same animal, there was at least a 1-week interval between scans.

EVALUATION

The calculation of R_2^* values was carried out on a pixel-by-pixel basis by fitting the gradient echo signal decay to a first-order model $\ln(S) = \ln(S_0) - R_2^* \text{TE}$. The corresponding percentage signal changes, $\Delta S/S$, reported in our activation studies are determined from ΔR_2^* as the values that would have been observed at a representative single echo time of TE = 45 ms (Blamire et al. 1992; Menon et al. 1993). The latter time is chosen to match the relaxation time T_2^* in brain tissue for maximum contrast [assuming the signal intensity S is maintained constant across studies ($\Delta S/S \approx -\Delta R_2^* \text{TE}$)]. While R_2^* itself does not report on the BOLD effect directly (only the temporal contrast due to changes ΔR_2^* does), repeatability in the measurement of baseline R_2^* values in specific brain regions of a given animal helps ensure consistency during replicate scans over a period of months. However, the baseline R_2^* in some tissues, such as the globus pallidus, does change slowly over a period of years due to metal deposition (e. g., iron) with aging. While there are differences between brain areas in their baseline R_2^* value, we have not seen a significant correlation between baseline and drug-evoked changes (ΔR_2^*). Thus, quantitative measures of changes in R_2^* can be detected regardless of baseline values. Additionally, any departure from a log-linear signal decay behavior can be mapped to help yield information about the intra- or extra-vascular origin of the signal (Chen et al. 1996). All of the analyses have been conducted using MAT:LAB software on Unix/Linux workstations (Mathworks, Natick, Mass., USA).

The fMRI data needed for computation of the R_2^* maps were acquired at a rate of one set consisting of three image slices every 30 s. Rigid-body motion between successive time points was not corrected since most effects were in the through-slice direction. Prior to administration of any drug, a total of 40 time frames were collected over 20 min for the baseline state. Following the injection of drug, an additional 40 images were collected to track the dynamic response. First, the time-course data for each pixel were adjusted by centering so as to have a zero mean during the baseline period comprising the first 40 time frames. The sample covariance matrix of dimension 80×80 was then computed from the measured R_2^* response for all image voxels in the brain, and the principal component time-course profiles were determined as the eigenvectors. The associated pixel-wise scores across the slice images represented the spatial modes/patterns.

Information in the navigator echo data from all three slices was used to detect line-to-line changes and sub-

sequently discard image time frames where motion had occurred during the acquisition. Additionally, formal statistical methods of influence analysis for PCA were used to flag and subsequently remove outlying observations that exerted undue influence and contributed disproportionately in the partitioning of variance (Andersen et al. 2002). These observations typically corresponded to spikes at points in the time series data and present as artifacts in the corresponding R_2^* maps. In a typical fMRI study of an awake rhesus monkey, on the order of 5–10 time frame observations out of a total of 80 would be detected by the navigator echo and 10–20 would be flagged as statistical outliers in the influence analysis for PCA. From the trimmed data set, the sample covariance matrix and principal component time course profiles were then recomputed yielding a far more robust representation.

The first principal component, which also represents the largest source of variance in the data, was then used to model the temporal response in each voxel across the brain image slices. This approach of filtering and fitting enables both an analysis of the dynamic nature of the temporal response to administration of a drug in a single animal and the development of an unbiased measure of activation that can be used in the analysis of data combined across multiple animals. From the filtered data, the change ΔR_2^* representing the fMRI activation response to a drug was determined as the difference between the mean R_2^* value across 20 images post drug administration during the period of peak response and the mean value within the 40 images acquired as baseline prior to administration.

MODIFICATIONS OF THE METHOD

Stefanacci et al. (1998) and Dubowitz et al. (2001) reported MRI studies in conscious monkeys.

Howell et al. (2001) described an apparatus and behavioral training protocol to conduct positron emission tomography (PET) neuroimaging in conscious rhesus monkeys reporting the development and standardization of PET neuroimaging protocols in conscious rhesus monkeys and their application to the characterization of the acute effects of cocaine on cerebral blood flow. Specific attention was devoted to the development of an effective and comfortable head restraint device to be used in the imaging of conscious monkeys. The restraint device was designed to attach to a standard primate chair to facilitate frequent immobilization. Subjects received extensive behavioral training prior to neuroimaging in order to ensure their comfort and minimize potential stress associated with

the imaging protocols. Functional changes in cerebral blood flow were characterized in three subjects with the positron-emitting tracer ^{15}O water following acute i.v. administration of cocaine. Regions of interest were defined on MRI scans with a high degree of accuracy.

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E.10.3.2

Functional NMRI Studies in the Brain of Common Marmosets

PURPOSE AND RATIONALE

Massacesi et al. (1995) and Genain and Hauser (1997) described actively and passively induced experimental autoimmune encephalomyelitis in common marmosets as a model of multiple sclerosis. ‘t Hart et al. (1998, 2004) used this model for detailed MRI studies with histopathological characterization of MRI-detectable white matter lesions.

PROCEDURE

Eleven marmosets (*Callithrix jacchus*) were used for this study. The body weight of the monkeys at the start

of the experiment ranged between 295 and 320 g. During the experiments, the monkeys were individually housed in spacious cages with padded shelter on the bottom. The daily diet consisted of commercial food pellets for non-human primates, supplemented with rice and fresh fruit. Drinking water was provided ad libitum

Induction of Experimental Autoimmune Encephalomyelitis (EAE)

Myelin was isolated from human brain white matter, which was kindly provided by Dr. Rivka Ravid of the Dutch Brain Bank (Amsterdam, The Netherlands). The myelin concentration in the stock solution was 30 mg/ml on a dry-weight basis and 1.3 mg/ml protein on a protein basis as measured according to Bradford (1976).

In a first group of five monkeys the myelin stock solution was emulsified in an equal volume of enriched complete adjuvant. Enriched complete adjuvant was prepared by mixing incomplete adjuvant (DIFCO Laboratories, Detroit, Mich., USA) with 6 mg/ml desiccated mycobacteria (*Mycobacterium tuberculosis*, H37A, DIFCO) followed by brief sonication. Under ketamine anesthesia, each monkey was injected intradermally on the back with 600 µl of emulsion divided over four spots, two in the inguinal and two in the axillary region. In addition, 1 ml phosphate-buffered saline (pH 7.4) containing 1010 heat-inactivated *Bordetella pertussis* particles was injected immediately after immunization and 48 h later.

In a second group of six monkeys, the original immunization protocol was modified in two respects. First, the *Mycobacterium* concentration in the antigen-adjuvant emulsion was reduced from 1.5 to 0.5 mg/ml to diminish the severity of ulceration around the injection sites. Secondly, *B. pertussis* administration as second adjuvant was omitted to obtain a milder EAE in which patterns of EAE reactivity can be associated with the presence of certain mhc alleles and cytokine profiles.

Clinical Diagnosis

The clinical course of EAE was recorded daily by a trained observer using semiquantitative scoring: 0 = no clinical signs; 0.5 = apathy, loss of appetite, altered walking pattern without ataxia; 1 = lethargy and/or anorexia; 2 = ataxia; 2.5 = para- or monoparesis and/or sensory loss and/or brainstem syndrome; 3 = para- or hemiplegia; 4 = quadriplegia; 5 = spontaneous death attributable to EAE. The highest per-day scores were averaged over 1 week. Moreover, each

monkey was weighed at least three times per week to obtain a more objective score of the clinical wellbeing.

MRI

MRI was performed at the Bijvoet Center of Utrecht University, the Netherlands. For each monkey, T2- and T1-weighted magnetic resonance (MR) images were recorded, the latter also with contrast enhancement after intravenous injection of gadolinium-diethylenetriamine-penta-acetic acid (DTPA) (triple dose). The time points for performing MRI after immunization were chosen during periods of clinically active EAE

In Vivo MRI

In preparation for the experiment, the monkeys were anesthetized with ketamine/Vetranquil (9/1 v/v). During scanning, each monkey was placed on a 37°C water-filled heating pad to prevent hypothermia. The head of the monkey was fixed in a custom-built stereotaxic apparatus made of metal-free plastics to ensure reproducible positioning in the magnetic field and to minimize movement artifacts. The stereotaxic apparatus was placed inside a saddle-type radiofrequency coil.

MRI was performed on a SISCO 200-MHz spectrometer (Varian, Palo Alto, Calif., USA) equipped with an actively shielded gradient (maximum gradient 3.2 G/cm, 33 cm inner diameter).

Postmortem MRI

T2-weighted MR images were recorded from formalin-fixed brains to enable the determination of the exact localization of the lesions that were detected *in vivo*. In both scannings the same orientation points were chosen for slice localization. Because movement artifacts were absent and long acquisition times can be used, images of very high contrast can be obtained.

Slice Orientation and Scanning Procedure

First, a sagittal scout scan was made. The posterior and anterior positions of the corpus callosum were chosen as orientation markers for precise localization of the slices for the *in vivo* and postmortem MRI. A T2-[echo time (TE)/repetition time (TR), 60/2500 ms-] weighted multislice scan (20 slices of 1 mm thickness) was obtained followed by a T1-weighted scan (TE/TR, 25/1000 ms) with the same spatial prescription. Gadolinium-DTPA (Magnevist; Schering, Berlin, Germany) was injected intravenously (0.3 mmol/kg)

and allowed to circulate for 10 min to ensure adequate distribution. Next, the T1-weighted MRI was repeated to attain a post-contrast data set. Each slice was recorded with a matrix of 256×128 data points and a field of view of 5×5 cm. The data set was analyzed on an Apple Macintosh Performa 630 using the public domain NIH Image program.

The T1-weighted MR images had an unexpected gray-white matter contrast. In images recorded with the NMR machines that are now used in clinical settings, which are 1.5 T or less, a contrast conversion of the white and gray matter is normally seen on T1-weighted versus T2-weighted images. In T1-weighted images the NMR signal of the white matter is hyperintense in comparison with that of the gray matter, whereas in T2-weighted images the white matter signal is hypointense compared with that of the gray matter.

In both the T1- and T2-weighted images recorded on our 4.7-T machine, white matter is hypointense as compared with gray matter, and lesions are visible as hyperintensities. This is not a unique feature of the marmoset brain, given that it was also observed in cat brains. The most likely explanation for this discrepancy is that with the high magnetic field used, T1 values of gray and white matter converge, whereas T2

values are unaffected by the strength of the field. In our T1-weighted images, the intensities of white and gray matter are most likely determined by the longer T2 value of gray matter and the different proton densities of both tissues. This phenomenon did not affect the detection of gadolinium enhancement, because even in long TR/short TE images ("proton-density" weighted) contrast enhancement can be observed.

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Chapter F

Drug Effects on Learning and Memory¹

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F.1 Introduction

It is easily understood that behavioral psychopharmacologies faced with the task of dealing with extremely complex behavioral disturbances. This holds true for both patient groups: young people with learning and memory problems and elderly patients with memory deficits. For the elderly, difficulties arise in designing appropriate animal models of human aging or the deficits occurring during human aging. One of the major problems for experimental behavioral pharmacology is whether or not old animals are the appropriate models. At the first view it seems obvious that the study of potential geronto-psychopharmacologic drugs should be performed in old animals. However, the problem is much more complicated. Laboratory animals are not a homogenous population, especially when old. Most of these old animals, which are one-third survivors of a population, have an individually different pathological history that is mostly unknown to the investigators. Some animals may be arthritic others may have bronchitis or cardiac deficiencies. If, for example, an arthritic rat is given a performance task associated with lever pressing, the animals may fail because of his rigid and painful joints and not because of a brain deficit or of the ineffectiveness of the test compound. Similar effects can be observed with old animals having a cataract in a visual discrimination task. Failure to perform a task may even be the result of both central and peripheral disturbances. Consequently it is impossible to describe the failure of one animal to perform the task to deficits in some parts of the brain or to pathological changes in the body.

Considerable evidence indicates that there are age-related changes in learning and memory (Gold and McGaugh 1975; Gold et al. 1975). Myslivecek (1997) described ontogeny of memory and learning in rats. For certain kinds of learning, there are specific developmental periods at which acquisition and retention are optimal; for example, imprinting in animals (Hess 1972; Scheich 1987) and language acquisition in humans (Lennenberg 1967). For other forms of learning and memory, acquisition and long-term retention in animals mature gradually with development (Campbell and Spear 1972). There is some evidence that

aged organisms have impaired memory function. For example, in humans, performance on recent memory tasks appears to decay at shorter training-test intervals and is more susceptible to retroactive interference in elderly subjects than in young adult subjects (Craik 1977; Kubanis and Zornetzer 1971). Recent memory also appears to be deficient in aged non-human primates (Bartus et al. 1980).

Although drug discovery is based upon many factors, animal models provide a crucial part in identifying chemical compounds with potential for clinical efficacy. A number of important animal models of human disease have come into existence based upon "serendipitous" discoveries of their ability to identify chemicals with a particular set of therapeutic actions. However, in the absence of truly effective therapeutic agents for age-associated dementia to serve as standards, the development of appropriate animal models for dementia represents a serious challenge. In the case of Alzheimer's disease or age-associated memory impairment, successful development of animal models may well depend upon our ability to accurately reproduce specific pathophysiological or etiologic factors.

It is generally agreed that an ideal animal model of human dementia would exhibit some or all the behavioral and neurological dysfunctions known to be associated with the clinical disorder, as well as the pathological processes leading to the demented condition. An early approach was the measurement of the spinal cord fixation time in rats as described by Giurgea and Mouravieff-Lesiusse (1971), Giurgea and Salama (1977).

McDonald and Overmier (1998) presented a critical review of animal models of the mnemonic impairments in Alzheimer's disease. Socci et al. (1995) could show that chronic antioxidant treatment improves cognitive performance of aged rats. Several authors discussed chronic alcohol intoxication in rats and mice as a model for neurodegeneration (Ahrendt et al. 1988; Fadda and Rossetti 1998; Raghavendra and Kulkarni 2001; Wozniak et al. 2004).

Miller et al. (1999) and McClearn (1999) discussed the use of inbred strains for gerontological research. There are limitations on research with uniform genotypes and advantages of heterogeneous populations. However, for specific purposes, the uniformity and stability of inbred strains are extremely valuable attributes.

Since cholinesterase inhibitors, such as physostigmine (Christie et al. 1981; David and Mohs 1982; Thal et al. 1983) and tacrine (Summers et al. 1986) have been shown to improve cognitive function in

patients with Alzheimer's type dementive disorders and based on analytical data in brains of aged subjects, the cholinergic hypothesis of geriatric memory dysfunction has been established. Numerous attempts have been made to study the influence of potential drugs on the central cholinergic system, e. g., inhibition of acetylcholinesterase activity in rat striatum *in vitro*, differentiation of this activity versus the inhibition of butyrylcholinesterase activity, *ex vivo* cholinesterase inhibition in rat striatum, release of acetylcholine from rat brain slices after electrical stimulation, binding to muscarinic cholinergic receptors in rat forebrain, stimulation of phosphatidyl-inositol turnover via muscarinic receptors, binding to nicotinic cholinergic receptors in rat frontal cortex. Presynaptic cholinergic markers include choline acetyl-transferase and acetylcholinesterase activities, high-affinity transport of choline, acetylcholine synthesis, and muscarinic receptor binding. The high-affinity transport of choline is influenced by a cholinotoxin, ethylcholine aziridinium (AF64A) that induces *in vivo* a persistent central cholinergic hypofunction of presynaptic origin (Fisher and Hanin 1986).

Changeux et al. (1998) discussed the role of nicotinic acetylcholine receptors in the brain in learning and reinforcement.

These approaches may give hints for the discovery of new antidementic drugs. The "cholinergic hypothesis" (Bartus et al. 1982, 1987) is not unequivocal in the pathophysiology of Alzheimer's disease (Francis et al. 2005). The role of other transmitters has to be studied (Nordberg 1990). *In vitro* and *in vivo* studies have to be performed in parallel in order to find new approaches to treat Alzheimer's disease or Alzheimer's type dementia.

The **amyloid cascade hypothesis** has gained much interest (Thorsett and Latimer 2000; Yamada and Nabeshima 2000; Emre and Qizilbash 2001; Sommer 2002; Lichtenthaler and Haass 2004).

As early as 1907, Alzheimer described the appearance of plaques and neurofibrillary tangles (Alzheimer 1907). Senile plaques are extracellular insoluble, cationophilic protein aggregates composed of amyloid β , a 39- to 43-amino-acid peptide. Neurofibrillary tangles are intracellular lesions consisting of paired helical filaments from the hyperphosphorylated cytoskeletal protein τ , which is a microtubule-associated protein that is involved in microtubule assembly and stabilization. In Alzheimer's disease, τ is abnormally phosphorylated, aggregates into paired helical filaments, and loses its ability to stabilize axonal microtubules (Mandelkowitz and Mandelkowitz 1998).

Goedert and Hasegawa (1999) discussed the tauopathies as an experimental animal model for Alzheimer's disease and other dementing disorders.

The discovery of autosomal dominant mutations in the gene encoding the amyloid precursor protein that were causal for early-onset familial Alzheimer's disease strongly suggested amyloid β to be the prime suspect in the pathogenesis of Alzheimer's disease, resulting in the formulation of the amyloid cascade hypothesis (Hardy and Higgins 1992).

In line with the amyloid cascade hypothesis, a strategy for disease-modifying pharmacological intervention would be the selective inhibition of β - and γ -secretase, the proteases that process amyloid precursor protein to amyloid β .

Bartzokis (2004) presented age-related myelin breakdown as a hypothetical model of cognitive decline and Alzheimer's disease.

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F.2 In Vitro Methods

F.2.0.1 In Vitro Inhibition of Acetylcholine-Esterase Activity in Rat Striatum

PURPOSE AND RATIONALE

The purpose of this assay is to screen drugs for inhibition of acetylcholine-esterase activity. Inhibitors of this enzyme may be useful for the treatment of Alzheimer's disease.

Acetylcholinesterase (AChE), which is sometimes called true or specific cholinesterase, is found in nerve cells, skeletal muscle, smooth muscle, various glands and red blood cells (Nachmansohn and Rothenberg 1945; Koelle et al. 1950; Ellman et al. 1961). AChE may be distinguished from other cholinesterases by substrate and inhibitor specificities and by regional distribution. Its distribution in brain roughly correlates with cholinergic innervation and subfractionation shows the highest level in nerve terminals.

It is generally accepted that the physiological role of AChE is the rapid hydrolysis and inactivation of acetylcholine. Inhibitors of AChE show marked cholinomimetic effects in cholinergically-innervated effector organs (Taylor 1996) and have been used therapeutically in the treatment of glaucoma, myasthenia gravis and paralytic ileus. However, recent studies (Christie et al. 1981; Summers et al. 1981; Davies and Mohs 1982; Atak et al. 1983) have suggested that AChE inhibitors may also be beneficial in the treatment of Alzheimer's dementia.

Augustinsson (1971) reviewed a number of methods for assaying cholinesterase activity and concluded that the method described by Ellman et al. (1961) was one of the best. The method described is a modification of Ellman's procedure.

PROCEDURE

Reagents

- 0.05 M Phosphate buffer, pH 7.2
 - 6.85 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ /100 ml distilled H_2O
 - 13.40 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ /100 ml distilled H_2O
 - add a) to b) until pH reaches 7.2
 - Dilute 1:10
- Substrate in buffer
 - 198 mg acetylthiocholine chloride (10 mM)

- b) q.s. explain to 100 ml with 0.05 M NaH₂PO₄, pH 7.2 (reagent 1)
3. DTNB in buffer
- a) 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
- b) q.s. to 100 ml with 0.05 M NaH₂PO₄, pH 7.2 (reagent 1)
4. A 2 mM stock solution of the test drug is made up in a suitable solvent and q.s. to volume with 0.5 mM DTNB (reagent 3). Drugs are serially diluted (1:10) such that the final concentration (in cuvette) is 10⁻⁴ M and screened for activity. If active, IC₅₀ values are determined from the inhibitory activity of subsequent concentrations.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, corpora striata dissected free, weighed and homogenized in 19 volumes (approximately 7 mg protein/ml) of 0.05 M NaH₂PO₄, pH 7.2 using a Potter-Elvehjem homogenizer (Kontes, Vineland, NJ). A 25 µl aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and reincubated for 10 min at 37°C.

Assay

Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for IC₅₀ determinations and for measuring kinetic constants.

Reagents are added to the blank and sample cuvettes as follows:

- Blank:** 0.8 ml PO₄ buffer/DTNB
0.8 ml buffer/Substrate
- Control:** 0.8 ml PO₄ buffer/DTNB/Enzyme
0.8 ml PO₄ buffer/Substrate
- Drug:** 0.8 ml PO₄ buffer/DTNB/Drug/Enzyme
0.8 ml PO₄ buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kin-data program available on kinetics soft-pac module. This program (Beckman DU-50 series spectrophotometer, kinetics Soft-PacTM module operation instructions: 1-7 also calculates the rate of absorbance change for each cuvette.

EVALUATION

For IC₅₀ determinations: Substrate concentration is 10 mM diluted 1:2 in an assay yielding a final con-

centration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration

$$\% \text{ Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

IC₅₀ values are calculated from log-probit analysis.

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F.2.0.2**In Vitro Inhibition of Butyrylcholine-Esterase Activity in Human Serum****PURPOSE AND RATIONALE**

This assay can be used in conjunction with the acetylcholine-esterase assay to determine the enzyme selectivity of various cholinesterase inhibitors.

Butyrylcholine-esterase (BChE), which is sometimes called pseudocholinesterase, preferentially hydrolyzes butyrylcholine. This enzyme is found in the highest amounts in serum, but its physiological role is not known (Chemnitiu et al. 1983; Walker and Mackness 1983). Ethopropazine and tetra-isopropyl pyrophosphoramidate (ISO-OMPA) are selective inhibitors of butyrylcholinesterase. In an *ex vivo* experiment with the selective BChE inhibitor, ISO-OMPA, it was shown that inhibition of butyrylcholinesterase was not correlated with any significant acute cholinomimetic effects.

PROCEDURE**Reagents**

- 0.05 M phosphate buffer, pH 7.2
 - 6.85 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ /100 ml distilled H_2O
 - 13.40 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ /100 ml distilled H_2O
 - Add (a) to (b) until pH reaches 7.2
 - Dilute 1:10
- Substrate in buffer
 - 225.8 mg s-butrylthiocholine chloride
 - q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)
- DTNB in buffer
 - 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
 - q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)
- A 2 mM stock solution of the test drug is made up in a suitable solvent and q.s. to volume with 0.5 mM DTNB (reagent 3). Drugs are serially diluted (1:10) such that determined from the inhibitory activity of subsequent concentrations.

Enzyme Preparation

A vial of lyophilized human serum (Precilip, Biodynamics, Houston, Texas) is reconstituted in 3 ml of distilled water. A 25 ml aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and pre-incubated for 10 min at 37°C.

Assay

Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for

IC_{50} determinations and for measuring kinetic constants.

Reagents are added to the blank and sample cuvettes as follows:

- Blank:** 0.8 ml PO_4 buffer/DTNB
0.8 ml buffer/Substrate
- Control:** 0.8 ml PO_4 buffer/DTNB/Enzyme
0.8 ml PO_4 buffer/Substrate
- Drug:** 0.8 ml PO_4 buffer/DTNB/Drug/Enzyme
0.8 ml PO_4 buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.

EVALUATION

For IC_{50} determinations: Substrate concentration is 10 mM diluted 1:2 in assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration

$$\% \text{ Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

IC_{50} values are calculated from log-probit analysis.

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F.2.0.3**Ex Vivo Cholinesterase Inhibition****PURPOSE AND RATIONALE**

This assay is used to determine the dose-response relationship and duration of action of cholinesterase inhibitors *in vivo*.

Cholinesterase inhibitors, including physostigmine (Christie et al. 1981; David and Mohs 1982; Thal et al. 1983) and tacrine (Summers et al. 1986) have been shown to improve cognitive functions in Alzheimer's disease. Physostigmine is a potent, but nonselec-

tive inhibitor of cholinesterase (Taylor 1996) and has a short duration of action. Tacrine also inhibits both acetylcholine-esterase (true) and butyrylcholine-esterase (pseudo), but is more potent as an inhibitor of the pseudo-enzyme (Heilbronn 1961).

The mechanism of inhibition of these two drugs is quite different. Physostigmine is a competitive inhibitor and blocks the active site of the enzyme by carbamylation of a serine hydroxyl group at the esteratic site of the enzyme (Taylor 1996; O'Brien 1969). This covalently bound carbamyl group then dissociates from the enzyme much more slowly than the acetyl group left by the natural substrate, but the inhibition is not irreversible like that of the organophosphates. The inhibition characteristics of physostigmine, i. e., submicromolar affinity for the enzyme and covalent binding of the inhibiting group, are ideal for *ex vivo* studies. Tacrine, however, is a mixed competitive inhibitor of cholinesterase (Heilbronn 1961), with lower apparent affinity than physostigmine for the enzyme (based on IC_{50} values at saturated substrate concentrations). Tacrine binds to the anionic site of cholinesterase through weak hydrophobic interactions (Steinberg et al. 1975).

Ideally, a dose-response for cholinesterase inhibition is determined first. Then a dose which gives a reasonable effect (>50% inhibition if possible) is chosen to do the time-course experiment. The effects on brain acetylcholinesterase activity are examined in striatal tissue, using 5 mM acetylthiocholine as a substrate (Ellman et al. 1961). Effects on butyrylcholinesterase activity may be determined in plasma samples, with 5 mM butylthiocholine used as a substrate.

PROCEDURE

Reagents

1. 0.05 M Phosphate buffer, pH 7.2
 - a) 6.85 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ /100 ml distilled H_2O
 - b) 13.40 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ /100 ml distilled H_2O
 - c) add a) to b) until pH reaches 7.2
 - d) Dilute 1:10
2. DTNB in buffer
 - a) 19.8 mg 5,5-dithiobisnitrobenzoic acid (DTNB) (0.5 mM)
 - b) q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)
3. Substrate in buffer
 - a) 198 mg acetylthiocholine chloride (10 mM)
 - b) q.s. to 100 ml with 0.05 M phosphate buffer, pH 7.2 (reagent 1)

Drug Treatment

Groups of four male Wistar rats are dosed i.p. or p.o. with vehicle or the test drug. For the initial dose response study, the rats are given varying doses of drug based on toxicity reported in primary overt effects testing and sacrificed at either 30 min or 1 h after dosing. The animals are observed and the occurrence of cholinergic signs is noted (piloerection, tremors, convulsions, salivation, diarrhea and chromodacryorrhea). For the time-course study, a dose of the test drug is given which gave significant inhibition of cholinesterase activity.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, corpora striata dissected free, weighed and homogenized in 4 volumes of 0.05 M phosphate buffer, pH 7.2 using a Potter-Elvehjem homogenizer (Kontes, Vineland, NJ). A 12.5 ml aliquot of the homogenate is added to 1 ml 0.05 M phosphate buffer, pH 7.2/DTNB (reagent 2).

Assay

1. Enzyme activity is measured with the Beckman DU-50 spectrophotometer. Reagents are added to the blank and sample cuvettes as follows:
 - Blank:** 0.8 ml PO_4 buffer/DTNB (reagent 2)
0.8 ml PO_4 buffer/Substrate (reagent 3)
 - Control:** 0.8 ml PO_4 buffer/DTNB/Enzyme from control animal
0.8 ml PO_4 buffer/Substrate
 - Drug:** 0.8 ml PO_4 buffer/DTNB/Enzyme from treated animal
0.8 ml PO_4 buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kinetics program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.

2. Substrate concentration is 10 mM diluted 1:2 in the assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration.

EVALUATION

The percent inhibition at each dose or time is calculated by comparison with the enzyme activity of the

vehicle control group.

$$\% \text{ Inhibition} = \frac{\text{slope control} - \text{slope drug}}{\text{slope control}} \times 100$$

Ex vivo time course experiments for physostigmine

| Physostigmine, 0.3 mg/kg, i.p. | | |
|--------------------------------|-----------------------|-------------------|
| Time (h) | % Inhibition striatum | Cholinergic signs |
| 0.25 | 48.6 | P,T,D |
| 0.5 | 28.5 | P,T,D |
| 1.0 | 27.0 | P,T |
| 2.0 | 7.6 | P,T |
| 4.0 | 1.4 | P,T |

P = piloerection, T = tremors, D = diarrhea.

MODIFICATIONS OF THE METHOD

Antagonism of physostigmine-induced lethality in mice has been used by Gouret (1973) as a general indicator of central or peripheral anticholinergic activity. A low dose of physostigmine can be used for detecting procholinergic activity.

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F.2.0.4

Molecular Forms of Acetylcholinesterase from Rat Frontal Cortex and Striatum

PURPOSE AND RATIONALE

Different molecular forms of acetylcholinesterase can be isolated from animal tissues after solubilization in buffers containing various detergent and salt concentrations. The number of forms isolated, their relative amounts and molecular characteristics depend on the tissue source and the conditions used for solubilization of the membrane bound enzyme (McIntosh and Plummer 1973; Reiger and Vigny 1976; Trevor et al. (1976). Bon et al. (1979) have classified these forms as globular (G_1 , G_2 and G_4) and asymmetric (A_4 , A_8 and A_{12}), where the subscripts indicate the number of catalytic subunits. The G_1 and G_4 forms, having sedimentation coefficients of approximately 4S and 10S, respectively, are the major forms contained in bovine caudate nucleus (Grassi et al. 1982). Under conditions of high salt concentrations and detergent, AChE is quantitatively extracted from rat brain, with 10S form being the predominant component (Reiger and Vigny 1976). After solubilization, the molecular forms may be separated according to sedimentation properties by density gradient centrifugation, molecular weight by gel filtration or by electrophoretic mobility.

Although most of these studies on molecular forms of AChE have focused on the physical differences, Chan et al. (1972) reported some difference in sensitivity of the low molecular weight form to physostigmine and fluoride ion, while Lenz and Maxwell (1981) showed differential sensitivity to soman of forms separated by isoelectric focusing. Studies showing selective increases in the 10S form during development (Muller et al. 1985) and selective loss in Alzheimer's disease (Atack et al. 1983) suggest that this form of the enzyme may be developmentally and functionally more important. Cortical and striatal areas show different patterns of cholinergic innervation; the cortex having primarily extrinsic innervation, while the striatal cholinergic pathways are predominantly intrinsic (Ceullo and Sofroniew 1984).

The purpose of this procedure is to determine the effects of various cholinesterase inhibitors on the two major molecular forms of acetylcholinesterase isolated from rat striatum and cerebral cortex.

PROCEDURE

The procedure is divided into three main parts:

- I. Preparation and isolation of molecular forms of AChE,
- II. Assays for the marker enzymes,
- III. Enzyme inhibition studies.

I.a Preparation of molecular forms of AChE

Male Wistar rats (200–250 g) are sacrificed, their brains rapidly removed and frontal cortices or corpora striata removed. The brain areas are weighed and homogenized in 5 volumes (wt/vol) of 10 mM phosphate buffer, pH 7.1, containing 1 M NaCl and 1% Triton X-100, except where indicated. The homogenates are centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant is aspirated and marker enzymes for 16S (*E. coli* β -galactosidase), 11.3S (bovine catalase) and 4.8S (horse liver alcohol dehydrogenase) fractions are added. The supernatant is then centrifuged at 37,000 rpm (140,000 *g* max) for 17.5 h in a Beckman L5–65 ultracentrifuge with a SW-60 rotor. 15-drop fractions are collected for each centrifuge tube and assayed for protein, β -galactosidase, catalase, alcohol dehydrogenase and acetylcholinesterase activity. In addition, butyrylcholinesterase can be measured.

A 400 μ l sample of the 20,000 *g*-supernatant is carefully layered on top of a 5–20% continuous sucrose gradient. This gradient is made up in a centrifuge tube from 1.65 ml of 20% sucrose and 1.65 ml of 5% sucrose in homogenizing buffer by means of a gradient maker. A 50% sucrose cushion (0.5 ml) is placed at the bottom of the tube.

Fractions are collected from the bottom of the tube, i.e., the densest fractions are collected first. Each fraction is 15 drops or approximately 24 fractions are collected per centrifuge tube.

I.b Analysis of fractions

β -Galactosidase, catalase and alcohol dehydrogenase are determined by enzymic activity (Hestrin et al. 1955; Chance and Maehly 1955; Bonnichsen and Brink 1955). Protein concentrations are determined by the method of Lowry et al.

(1951). Acetylcholinesterase activity or butyrylcholinesterase activity are determined by a modification of the method of Ellman et al. (1961). Briefly, 10 μ l aliquots of the fractions are added to 0.25 mM dithionitrobenzoic acid (DTNB) and 5 mM acetylthiocholine or 5 mM butyrylthiocholine in 0.05 M phosphate buffer, pH 7.2 and the absorbance is measured at 412 nm. Fractions of peak acetylcholinesterase activity are characterized by their sedimentation characteristics relative to the marker enzymes and peak fractions are pooled for enzyme inhibition studies or determination of kinetic constants.

II. Assays for marker enzymes

A Equine liver alcohol dehydrogenase (ADH), sedimentation coefficient 4.8 S

1. Enzyme: alcohol dehydrogenase from equine liver, crystallized and lyophilized (Sigma Chem. Co.)
2. Reagents:
 - a) β -nicotinamide adenine dinucleotide (NAD) (Sigma Chem. Co.)
 - b) 0.1 M glycine-NaOH buffer, pH 9.6
 - c) absolute ethanol
 - d) buffer-substrate-NAD mixture:
875 μ l NAD+875 μ l ethanol+18.75 ml
0.1 M glycine-NaOH buffer, pH 9.6
3. Assay
10 μ l enzyme fraction and
850 μ l mixture (reagent 2d)
are incubated for 5 min at room temperature. The reaction is stopped by adding:
300 μ l 1.5 M ZnSO₄
Absorbance is read at 340 nm and enzyme units are determined from a standard curve using values of 1.25, 2.5, 5, 10 and 20 mU of ADH.

B Bovine liver catalase, sedimentation coefficient 11.3 S

1. Enzyme: catalase from bovine liver, purified powder (Sigma Chem. Co., C-10)
2. Reagents:
 - a) 30% hydrogen peroxide
 - b) 0.05 M sodium phosphate buffer, pH 7.0
 - c) mixture: 111 μ l 30% hydrogen peroxide + 100 ml buffer, yielding 0.033% peroxide.
3. Assay
10 μ l enzyme fraction
2990 μ l peroxide-buffer mixture (reagent 2c)

Wavelength is set to 240 nm, absorbance is adjusted to 0.480 units. The amount of time is recorded for absorbance to decrease from 0.450 to 0.400. This corresponds to 3.45 μmol of hydrogen peroxide in 3 ml solution. Total catalase activity in 3 ml is 3.45 $\mu\text{Mol}/\text{min}$.

C *E. coli* β -galactosidase, sedimentation coefficient 16.0 S

1. Enzyme: β -galactosidase from *E. coli*, grade VI, partially purified, lyophilized (Sigma Chem. Co)
2. Reagents:
 - a) substrate: 15 mg/ml O-nitrophenyl- β -D-galacto-pyranoside (Sigma Chem. Co.) in water
 - b) 0.6 M Na_2CO_3 , pH 7.25
 - c) 1 M NaCO_3
3. Assay

10 μl enzyme fraction,
150 μl 0.6 M phosphate buffer, pH 7.25, and
50 μl O-Nitrophenyl- β -D-galactopyranoside (12 mM in assay) are incubated for 25 min at 30°C. The reaction is stopped by adding:
500 μl 1 M Na_2CO_3 , 1.75 ml water.
Absorbance is read at 420 nm and enzyme units are determined from a standard curve using values of 0.015, 0.030, 0.525, 0.125 and 0.250 U of β -galactosidase.

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III. Enzyme inhibition studies

For the enzyme inhibition studies, 25 μl aliquots of the enzyme preparation are preincubated with varying concentrations of the inhibitor for 10 min at 25°C and acetylcholinesterase activity is determined as previously described.

EVALUATION

Values for the IC_{50} are determined by log-probit analysis of the inhibition data using six to seven concentrations of the inhibitor and represent the means of 3 separate experiments.

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F.2.0.5

Release of [^3H]ACh and Other Transmitters from Rat Brain Slices

PURPOSE AND RATIONALE

Electrically stimulated release of [^3H]ACh is used as a biochemical screen for agents which may possibly enhance or inhibit release of [^3H]ACh through a direct muscarinic interaction or other indirect interactions.

Muscarinic autoreceptors have been shown to have a role in the regulation of ACh release in several areas of the CNS (Hadhazy and Szerb 1977; DeBellerocche and Gardiner 1982; Strittmatter et al. 1982; James and Cubeddu 1984). Direct stimulation of muscarinic receptors with muscarinic agonists or indirect stimulation with acetylcholinesterase inhibitors decreases ACh release evoked by either increased potassium concentration or electrical stimulation. Muscarinic antagonists can either block their inhibition, or, under certain conditions, enhance ACh release (James and Cubeddu 1987; Sethy et al. 1988). Furthermore, other

neurotransmitters, most notably 5-HT and DA, can also inhibit [³H]ACh release via interaction with 5-T₂ and D₂ heteroreceptors (Robinson 1983; Jackson et al. 1988; Muramatsu et al. 1988; Drukarch et al. 1989) and this inhibition can be reversed by the appropriate receptor antagonists. A compound's effect on [³H]ACh release may provide evidence for a wide variety of releasing activities.

The advantages of using the electrically stimulated release technique on tissue slices have been described by Zahniser et al. (1986). This technique measures only presynaptic effects of test compounds.

PROCEDURE

This assay is based on the methods described by James and Cubeddu (1984, 1987).

Reagents

1. Krebs-Henseleit bicarbonate buffer, pH 7.4 (KHBB)

Make a 2 liter batch, containing the following salts:

| | | |
|---------------------------------------|---------|----------|
| NaCl | 13.84 g | 118.4 mM |
| KCl | 0.70 g | 4.7 mM |
| MgSO ₄ · 7H ₂ O | 0.58 g | 1.2 mM |
| KH ₂ PO ₄ | 0.32 g | 2.2 mM |
| NaHCO ₃ | 4.20 g | 24.9 mM |
| CaCl ₂ | 0.28 g | 1.3 mM |
| Prior to use, add: | | |
| Dextrose | 4.00 g | 11.1 mM |

Aerate for 60 min with 95% O₂/5% CO₂ on ice and check pH.

2. [Methyl-³H]-choline chloride (80–90 Ci/mmol) is obtained from New England Nuclear. The final desired concentration of [³H]choline is 100 nM. Add 0.25 nmol of [³H]choline to 2.5 ml KHBB.
3. For most assays, a 10 mM stock solution of the test compound is prepared in a suitable solvent and diluted such that the final concentration in the assay is 10 μM. Higher or lower concentrations may be used depending on the potency of the test compound.
4. Hemicholinium-3 or HC-3 (Sigma): Make a 10 mM stock solution in H₂O. Two milliliters of this stock are then diluted to one liter in KHBB to give a final concentration of 20 μM.

Tissue Preparation

Male Wistar rats (100–150 g) are decapitated, and cortical, striatal, or hippocampal tissue removed on ice and 0.4 mm slices are prepared with a McIlwain tissue slicer. The slices are made individually, placed in

cold, oxygenated buffer (10–20 ml) and incubated at 35°C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 2.5 ml of cold oxygenated buffer is added, and enough [³H]choline to bring the final concentration to 100 nM. This is then incubated and shaken for 60 min at 35°C under oxygen. After this step, the buffer is decanted and the “loaded” slices are immediately placed on nylon mesh in the stimulation chambers.

Assay

Control buffer is pumped through the chamber for 42 min at a flow rate of 0.7 ml/min, to establish a stable baseline. Under these conditions released [³H]ACh is subject to hydrolysis by acetylcholinesterase. The perfusion buffer is changed to fresh KHBB containing 20 μM HC-3. The potent choline uptake inhibitor HC-3 is included to prevent the re-uptake of any [³H]choline formed from the hydrolysis of released [³H]ACh. This maintains the stoichiometry of the stimulated release. The evoked release has been shown to be mostly [³H]ACh rather than [³H]choline, whereas spontaneous release under control, drug-free conditions is mostly [³H]choline (Richardson and Szerb 1974; Szerb et al. 1977; Supavilai and Karobath 1985; Saijoh et al. 1985; Nishino et al. 1987).

SPECIAL CONDITIONS

1. Stimulation parameters are set at 2 Hz (2 ms duration) for 120 s, with 1 ms delay and voltage setting of 750 SIU (250 Ω). Agonists are more potent modulators of [³H]ACh release at low stimulation frequencies (5).
2. For striatal slices, 2 μM sulpiride is present in the buffer to prevent DA inhibition of ACh release.
3. In some experiments, 5 μM methysergide is present in the buffer to prevent the serotonergic inhibition of ACh release mediated by 5-HT₂ receptors.
4. In order to determine the muscarinic regulation of [³H]ACh release, 10 μM atropine can be included in some experiments.
5. In some experiments, physostigmine is added to the perfusion buffer. This causes a marked inhibition of stimulated release via feedback at pre-synaptic receptors. Under these conditions, receptor antagonists enhance [³H]ACh release.

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, 200 ml of 20% methanol in distilled water and again with distilled water for at least 20 min.

EVALUATION

After conversion of dpm, percent fractional release is calculated for each fraction, using a Lotus program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue. "Spontaneous Release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) values are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S_2/S_1 ratios. To normalize the data, drug effects can be estimated by first calculating S_2/S_1 values for control and drug-treated slices and then expressing the S_2/S_1 value for the drug-treated slices as a percentage of the S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from the same animal.

MODIFICATIONS OF THE METHOD**Release of Other Neurotransmitters
From Brain Tissue in Vitro**

Several authors (Harms et al. 1979; de Belleruche and Gardiner 1982; James and Cubeddu 1984; Raiteri et al. 1984; Zahniser et al. 1986; Smith et al. 1984, 1994) studied the release of neurotransmitters from brain tissue *in vitro*.

Raiteri et al. (1974) described a simple apparatus for studying the release of neurotransmitters from synaptosomes.

De Boer et al. (1988) determined the release of noradrenaline and serotonin in synaptosomes from rat cerebral cortex.

Saijoh et al. (1985) studied the influence of hypoxia on release and uptake of the neurotransmitters dopamine and acetylcholine in guinea pig striatal slices.

PROCEDURE**[³H] Norepinephrine Release from Cortical Slices**

Cortical slices (0.4 mm) from male Wistar rats are preincubated in Krebs buffer saturated with 95% O₂/5% CO₂, pH 7.4 for 30 min at 35°C, and then incubated in fresh buffer containing 25 nM [³H]NE (35 Ci/mmol) for 30 min at 35°C. The slices are then placed in glass superfusion chambers containing platinum electrodes and perfused at 0.75 ml/min. Fractions are collected at 7 min intervals. Slices are electrically stimulated with unipolar pulses (15–30 mA) of 2 ms duration at 5 Hz for 60 s. Two rounds of stimuli are applied, sepa-

rated by 10 fractions. Test compound is applied at fraction 14 (28 min after the first stimulation).

The fractions collected are counted for tritium in 10 ml of Liqiscint scintillation fluid and corrected for quench. For measurement of remaining tritium, slices are dissolved overnight in 0.5 ml of Protosol, buffered with 1 ml of Tris HCl, and counted. Percent fractional release is defined as the ratio of tritium released versus the amount present in the tissue.

**[³H] Norepinephrine Release
from Cortical Synaptosomes**

Cortices from male Wistar rats are homogenized in 9 volumes of 0.32 M sucrose in a Potter-Elvehjem homogenizer and then centrifuged at 1000 g for 10 min at 4°C. The supernatant is recentrifuged at 17000 g for 20 min, and the pellet is resuspended in 0.32 M sucrose at the original volume.

The freshly prepared synaptosomes are incubated with 50 nM [³H]NE in Krebs buffer containing 10 mM pargyline for 10 min at 37°C. The ratio of buffer to tissue suspension is 80:20. The [³H]NE-loaded synaptosomes are then separated by centrifugation (17000 g for 20 min), washed with buffer containing pargyline, recentrifuged, and then finally resuspended in 0.32 M sucrose at their original volume. The assay mixture consists of 900 ml Krebs buffer containing 10 mM pargyline, 100 ml of [³H]NE-loaded synaptosomes, and 10 ml of vehicle or drug. This mixture is then vortexed and incubated for 5 min at either 37°C or 0°C (to define total versus non-specific release). After a 10 min centrifugation (3000 g), the pellets are solubilized in a Triton X-100/ethanol mixture and transferred to scintillation vials, and counted in 10 ml of Liqiscint.

The net disintegrations for the 37°C and 0°C incubations are calculated, and % increase values determined as [(control–drug)/control] × 100.

Several chemical methods to measure acetylcholine (Israël and Lesbats 1982; Damsa et al. 1985; Stadler and Nesselhut 1986), or catecholamines and serotonin (Wagner et al. 1979; Magnusson et al. 1980; Nielsen and Johnston 1982; Wagner et al. 1982) are available.

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F.2.0.6**[³H]Oxotremorine-M Binding to Muscarinic Cholinergic Receptors in Rat Forebrain****PURPOSE AND RATIONALE**

The muscarinic receptors are members of the superfamily of G-protein-coupled receptors. They are relatively abundant and mediate the diverse action of acetylcholine in the CNS, as well as throughout non-nervous tissues innervated by the parasympathetic nervous system. Five separate genes (m1–m5) encode muscarinic receptor proteins exhibiting the rhodopsin-like structural motif containing seven transmembrane domains. They show strong sequence homology with each other and with related G-protein-coupled receptors within the transmembrane spanning domains, but each receptor has unique amino acid sequences located at the extracellular amino end and in the third intracellular loop (Caulfield 1993; Jones 1993; McKinney 1993; Wess 1996).

The purpose of this assay is to determine the binding affinity of potential cholinomimetic drugs for muscarinic receptors in brain, using an agonist ligand.

Oxotremorine is a potent centrally and peripherally acting muscarinic cholinergic agonist (Chao et al. 1962; Bebbington et al. 1966), which has been shown to be active in isolated tissue preparations as well as *in vivo* (Rindahl and Jenden 1983). Both central and peripheral effects of oxotremorine are blocked by anti-muscarinic drugs such as atropine (Chao et al. 1962; Bebbington et al. 1966). Structural modification of the oxotremorine molecular yields compounds which are full agonists, partial agonists and antagonists at muscarinic receptors (Rindahl and Jenden 1983). Oxotremorine-M (oxo-M), a quaternary nitrogen analog of oxotremorine, is a full agonist for the phosphatidyl-inositol response, while oxotremorine is a partial agonist (Fisher et al. 1984). Both oxotremorine and oxo-M are full agonists for inhibition of adenylate cyclase (Ehlert 1985; Olanas et al. 1983; Brown and Brown 1984). Of the muscarinic agonists, oxotremorine is the most potent inhibitor of [³H]QNB binding, however, the *IC*₅₀ is still only in the micromolar range. The apparent low affinity of agonist competition for [³H]-antagonist binding sites is a common phenomena and is due to the existence of multiple agonist affinity states of the receptor, as described by Birdsall et al. (1978). For this reason, it is desirable to use an agonist ligand to measure the binding affinities of potential agonists.

Molecular methods have disclosed the existence of five muscarinic receptors which are coupled to differ-

ent second messenger systems. At least four of them are expressed as functional receptor proteins in the neocortex and hippocampus formation (McKinney and Coyle 1991).

PROCEDURE**Reagents**

- 0.5 M Tris buffer, pH 7.4
66.1 g of Tris HCl
9.7 g of Tris base
q.s. to 1 liter with deionized water
- 0.05 M Tris buffer, pH 7.4
(10-fold dilution of reagent 1)
- [Methyl-³H]-oxotremorine acetate (specific activity 83–85 Ci/mmol) is obtained from New England Nuclear.
For *IC*₅₀ determinations: [³H]Oxotremorine-M is made up to a concentration of 100 nM. Fifty μl of this solution is added to each assay tube (yields a final concentration of 5 nM).
- Atropine sulfate is obtained from Sigma Chemical Company. A 2 mM stock solution is made up in distilled water. Twenty μl is added to 3 tubes for determination of non-specific binding (yields a final concentration of 40 μM).
- A 0.5% (w/v) solution of polyethylene-imine is prepared in distilled water. GF/C filters are soaked in this solution for at least three hours at room temperature. This is done to reduce the binding of the ligand to the filter strips.
- Test compounds: For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-4} to 2×10^{-7} M.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The forebrains (all tissue forward of a vertical cut in front of the hypothalamus) are weighed (400–500 mg each) and homogenized in 10 volumes of 0.05 M Tris buffer, pH 7.4 (reagent 2) using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 1000g for 10 min. The supernatant is then centrifuged at 50,000g for 60 min. The supernatant from this centrifugation is discarded and the pellet resuspended in the original volume of 0.05 M Tris buffer, pH 7.4, on the Polytron to 100 mg/ml (wet weight). Specific binding is roughly 1% of total added and 50% of total bound.

Binding Assay

- 50 µl** 0.5 M Tris buffer, pH 7.4 (reagent 1)
380 µl H₂O
20 µl drug or 2 mM atropine
50 µl ³H-oxotremorine-M (reagent 3)
500 µl tissue

Tubes are vortexed and incubated at 30°C for 45 min (2). Bound [³H]-oxotremorine-M is captured by filtration under reduced pressure. The incubation mixture is diluted with approximately 4 ml ice-cold 0.05 M Tris buffer, pH 7.4 (reagent 2), then exposed to vacuum, and tubes washed once more with approximately 5 ml of reagent 2. The filters (GFC in 0.5% polyethylene-imine for more than 3 h, reagent 5) are then counted in 10 ml Liquiscint scintillation fluid.

EVALUATION

Specific binding is the difference between total bound (in presence of vehicle) and that bound in the presence of 40 µM atropine. Percent inhibition of specific [³H]-oxotremorine-M is calculated for each concentration of test drug and IC₅₀ values determined by computer-derived log probit analysis. The percent inhibition at each drug concentration is the mean of duplicate or triplicate determinations. Some day-to day variability is present in this assay, and IC₅₀ values should be confirmed by repeat analysis.

MODIFICATION OF THE METHOD

[³H]Pirenzepine has been used to identify muscarinic receptor subtypes in the brain (Watson et al. 1983a, b).

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F.2.0.7**[³H]N-Methylscopolamine Binding in the Presence and Absence of Gpp(NH)p****PURPOSE AND RATIONALE**

G-protein-linked muscarinic receptors are converted by guanine nucleotides from a high-affinity bind-

ing state to a low-affinity binding state for muscarinic agonists (Gilman 1986), while the binding of muscarinic antagonists to the receptor is not affected. The effects of guanine nucleotides on muscarinic agonist affinity are brain region- and temperature-dependent (Aronstram and Narayanan 1988). Therefore, incubation of cerebellar membranes with 50 μ M 5'-guanylylimidophosphate (Gpp(NH)p), the non-hydrolyzable analog of GTP, causes a shift to the right (decreased affinity) of the muscarinic agonist inhibition curves when 3 H-NMS is used as the ligand.

The assay differentiates the interaction of muscarinic agonists and muscarinic antagonists with 3 H-N-methylscopolamine (3 H-NMS)-labeled receptors in cerebellar tissue based on the selective effect of guanine nucleotides on the affinity of muscarinic agonists for the receptor.

PROCEDURE

The procedure is based on 3 H-NMS rat brain binding assay described by Aronstram and Narayanan (1988).

Reagents

1. 0.5 M Tris-HCl buffer, pH 7.4
2. 0.05 M Tris-HCl buffer, pH 7.4
3. 0.05 M Tris-HCl buffer, pH 7.4 + 2 mM MgCl₂
4. 0.05 M Tris-HCl buffer, pH 7.4 + 2 mM MgCl₂ + 100 μ M phenylmethylsulfonyl fluoride
5. Atropine sulfate is made up to 1 mM in distilled water and 20 μ l is added to a 2 ml reaction mixture. This yields a final concentration of 10 μ M. Atropine is used for nonspecific binding.
6. 5'-Guanylylimidodiphosphate (Gpp(NH)p) is made up to 2 mM in distilled water. The final concentration in the reaction mixture is 50 μ M.
7. 3 H-N-Methylscopolamine (NMS) is obtained from Amersham and diluted to 4 nM in distilled water. The final concentration in the reaction mixture is 0.1 nM.
8. Test compounds
For most assays, a 10 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10⁻⁴ to 10⁻⁷ M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. The cerebella are dissected, weighed and homogenized in 10 volumes of 0.05 M Tris-HCl

buffer, pH 7.4 + 2 mM MgCl₂ + 100 μ M phenylmethylsulfonyl fluoride (buffer 4), using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 20,000 *g* for 20 min. The pellet is resuspended in 10 volumes of 0.05 M Tris-HCl buffer + 2 mM MgCl₂ (buffer 3).

Binding Assay

| | |
|-------------------------------|------------------------------------------------------------------|
| 1000 μl | 0.05 M Tris buffer + 2 mM MgCl ₂ |
| 780 μl | H ₂ O |
| 20 μl | vehicle or 1 mM atropine or appropriate drug concentration |
| 50 μl | H ₂ O or Gpp(NH)p |
| 50 μl | [3 H]NMS |
| 100 μl | tissue suspension |

Tubes are incubated at 20°C for 90 min. Bound [3 H]NMS is captured by vacuum filtration. The filters are washed three times with 5 ml aliquots of 0.05 M Tris buffer, pH 7.4. Filters are counted in 10 ml Liquiscint scintillation fluid.

EVALUATION

Specific binding of [3 H]NMS is the difference between total bound (in the presence of vehicle) and that bound in the presence of 1 mM atropine. Percent inhibition of specific [3 H]NMS binding is calculated for each concentration of test drug and IC₅₀ values are determined by computer-derived log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

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F.2.0.8

Stimulation of Phosphatidylinositol Turnover in Rat Brain Slices

PURPOSE AND RATIONALE

The purpose of this assay is to determine the ability of test compounds to stimulate the turnover of phosphatidylinositol (PI) in brain tissue. This assay can be used to determine agonist activity at a number of CNS receptors known to be linked to the PI response. A major interest is the evaluation of muscarinic cholinergic receptors.

Receptor-activated hydrolysis of inositol phospholipids is now recognized as an important second messenger system for muscarinic, alpha-adrenergic, histaminergic, serotonergic, excitatory amino acid and various neuropeptide receptors (Berridge and Irvine 1984; Nahorski et al. 1986; Fisher and Agranoff 1987). Furthermore, the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) yields at least two important biologically-active intermediates (Hirasawa and Nishizuka 1985; Berridge 1987). These include 1,4,5-inositol trisphosphate (IP₃), which acts to mobilize Ca²⁺ from the endoplasmic reticulum and diacylglycerol, which activates protein kinase C (PKC). These responses are associated with many cellular responses such as stimulus-secretion coupling, stimulus-contraction coupling and cell proliferation. The exact mechanism for receptor-mediated turnover of cell membrane PI is not well understood, but it seems to involve coupling through a G-protein (Cockcroft and Gomperts 1985) and requires extracellular Ca²⁺ to activate phospholipase C (Fisher et al. 1989).

Although the muscarinic receptor-PI link has been known for some time (Hokin and Hokin 1955), recent advances in knowledge of receptor mechanisms and increased interest in the muscarinic receptor have stimulated considerable research in this area. Even though muscarinic agonists can be shown to be weak partial agonists or full agonists for this response (Fisher et al. 1983) and there are brain regional differences in sensitivity (Fisher and Bartus 1985); attempts to show receptor subtype selectivity for stimulation of PI turnover in either heart (Brown et al. 1985) or brain tissue (Fisher and Bartus 1985) have been disappointing. However, recent experiments using cells transfected with genomic clones for the various muscarinic receptors have had more success (Shapiro et al. 1988; Conklin et al. 1988). These studies show that the m₁, m₃ and the m₅ receptor subtypes are linked to PI turnover.

Stimulation of PI turnover by agents such as veratridine, batrachotoxin and ouabain (Gusovsky et al. 1986) show that there are also non-receptor mechanisms that can cause the stimulation of PI turnover.

PROCEDURE

Equipment and Materials

1. McIlwain tissue slicer
2. Disposable columns (Kontes, 200 mm)
3. Column rack (Kontes)
4. Disposable screw-cap tubes (Pyrex, 16 × 100 mm)
5. Disposable culture tubes (Fisher, 16 × 125 mm)

Reagents

1. Modified Krebs bicarbonate buffer

| | g/l | mM |
|---------------------------------------|------|-------|
| NaCl | 8.30 | 142.0 |
| KCl | 0.42 | 5.6 |
| CaCl ₂ | 0.24 | 2.2 |
| NaHCO ₃ | 0.30 | 3.6 |
| MgCl ₂ · 6H ₂ O | 0.20 | 1.0 |
| HEPES | 7.15 | 30.0 |
| Adjust pH to 7.4 with NaOH | | |
| d-Glucose ^a | 1.01 | 5.6 |

^aGlucose added just before incubation.

2. Concentrated Krebs buffer+LiCl (9-fold concentrated ions, 10-fold concentrated Li⁺), stock solution contains no glucose or Ca²⁺.

| | g/100 ml |
|---------------------------------------|-----------------------------------------------------------|
| NaCl | 6.88 (amount adjusted to correct for Li ⁺) |
| KCl | 0.38 |
| NaHCO ₃ | 0.27 |
| MgCl ₂ · 6H ₂ O | 0.18 |
| HEPES | 6.44 |
| LiCl | 0.42 |

CaCl₂ (11 mg) and d-glucose (45 mg) are added to 5 ml of concentrated buffer before incubation.

3. Dowex AG-1-X8 (100–200 mesh) formate form (Biorad)
4. [³H]Inositol (spec. act. 15 Ci/mmol) is obtained from American Radiolabeled Chemicals, Inc.
5. Myo-inositol (M.Wt. 180.2) is obtained from Sigma Chemical Co. A 5 mM solution is made (0.9 g/l).
6. 1 M Ammonium formate/0.1 M formic acid. Ammonium formate is obtained from Sigma Chemical Co.: 3.85 ml 99% formic acid + 63.1 g ammonium formate to 1 liter in H₂O.
7. CHCl₃/methanol (1:2, v/v).

Tissue Preparation

Male Wistar rats, approximately 6/assay.

1. Remove surface blood vessels by rolling brain on filter paper.
2. Remove cerebral cortex and gently scrape myelin layer off.
3. Prepare 350 × 350 micron tissue slices with the McIlwain tissue chopper and place slices in buffer at 37°C.

Incubate for 10 min.

4. Disperse slices by aspirating into 1-ml pipet (cut off tip).
5. Allow slices to settle, aspirate supernatant, add buffer and repeat until the supernatant is clear.
12. Put 0.7 ml of supernatant into scintillation vial, add 10 ml cocktail and count.

Assay: Prelabeled Method

1. Add to screw-cap tubes:
 - 50 μ l tissue slices
 - 350 μ l [3 H]inositol
2. Incubate for 90 min at 37°C under O₂
3. Add:
 - 50 μ l drug
 - H₂O to a final volume of 500 μ l
4. Incubate for 30 min at 37°C under O₂.
5. Stop the reaction by the addition of 1.5 ml of CHCl₃: MeOH. Place in ice bath.

Assay: Continuous Labeling Method

1. Add to screw-cap tubes:
 - 50 μ l tissue slices
 - 350 μ l [3 H]inositol
 - 50 μ l drug
 - H₂O to a final volume of 500 μ l
2. Incubate for 120 min. at 37°C under O₂.
3. Stop the reaction by the addition of 1.5 ml of CHCl₃: MeOH. Place in ice bath.

For antagonist inhibition studies, the antagonist is usually preincubated with the slices before the agonist is added.

Extraction of Total [3 H] Inositol Phosphates

1. Add 1 ml of CHCl₃ and 0.5 ml of H₂O to each tube. Cap and vortex.
2. Centrifuge at 3000 rpm for 10 min.
3. Aspirate the aqueous phase and place in culture tubes.
4. Add 1.7 ml of H₂O and heat in water bath at 55°C for 20 min.
5. Place samples in cold room overnight.
6. Add 0.5 ml of Dowex 50% slurry to each tube. Vortex 4 times.
7. Centrifuge at 3000 rpm for 10 min.
8. Aspirate.
9. Add 2.5 ml of 5 mM myo-inositol, let the resin settle and aspirate. Repeat 5 times.
10. Centrifuge at 3000 rpm for 10 min.
11. Add 1 ml of ammonium formate/formic acid, pH 4.8.

EVALUATION

The stimulation phosphatidylinositol turnover for each test compound is calculated as percent increase in total [3 H] inositol phosphates relative to the basal turnover rate of non-treated control brain slices. The EC₅₀ values for agonists are determined by log-probit analysis of these data. IC₅₀ values for antagonists are determined by log-probit analysis of the percent inhibition of stimulation by a full agonist.

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[³H]N-Methylcarbamylcholine Binding to Nicotinic Cholinergic Receptors in Rat Frontal Cortex

PURPOSE AND RATIONALE

Nicotinic acetylcholine receptors are a family of ligand-gated ion channels that are classified on the basis of their activation by nicotine, although acetylcholine is the endogenous ligand. These conductance channels for Ca²⁺, K⁺ and Na⁺ are pentameric in structure. They are members of a supergene family that also includes glycine, GABA_A, and 5-HT₃ receptors. α , β , γ , and δ subunits constitute a pentameric neuronal receptor resulting in various receptor subtypes (Alkondon and Albuquerque 1993; Sargent 1993). Ten α (α 1– α 10) and four β (β 1– β 4) subunits have been cloned from mammalian and avian sources, each of which has a structural motif of four transmembrane spanning domains M1–M4 of which M2 lines the channel (Gotti et al. 1997; Jensen et al. 2005).

Nicotinic cholinergic receptors are classified as ligand-gated ion channels (Le Novere et al. 2002; Jensen et al. 2005), and are found in skeletal muscle, autonomic ganglia and brain tissue. Nicotine itself has a variety of behavioral effects. Due to its rapid desensitization of the receptor, both stimulatory and depressant effects may result. Also, many of nicotine's effects are thought to be associated with release of neurotransmitter substances (Balfour 1982). Nicotine functions as a nicotinic cholinergic receptor agonist in the CNS and is thought to play a role in learning and memory (Clarke 1987; Levey 1996; Dajas-Bailador and Wonnacott 2004). Reductions in nico-

tinic binding sites were found in post-mortem tissues from Alzheimer's patients by four separate groups of investigators (Whitehouse et al. 1988; Nordberg and Winblack 1986; Araujo et al. 1988; Shimohama et al. 1986). Neuronal nicotinic acetylcholine receptors play a role in acute and chronic neurodegeneration (O'Neill et al. 2002).

Unconventional ligands and modulation of nicotinic receptors are discussed by Pereira et al. (2002).

The structure and function of the acetylcholine-binding protein (ACHB), a homolog of the ligand-binding domain of the nicotinic acetylcholine receptor, are described by Smit et al. (2003).

Unwin (2003) explored structure and action of the nicotinic acetylcholine receptor by electron microscopy.

Cognitive improvement in Alzheimer patients 30 min after a nicotine infusion was reported (Sunderland et al. 1988). Therefore nicotinic agonists may prove beneficial (Hogg and Bertrand 2004), however, clinical data are still quite limited. N-Methylcarbamylcholine (NMCC) is a nicotinic agonist which binds specifically and with high affinity to central nicotinic receptors and, like nicotine, causes an increase of acetylcholine release from certain cholinergic nerve terminals (Araujo et al. 1988; Lapchak et al. 1989). In addition, chronic nicotine treatment increases [³H] NMCC binding sites in several rat brain regions, as it does with [³H]nicotine and [³H]ACh labeled sites (Lapchak et al. 1989). This is due to the loss of presynaptic nicotinic autoreceptor function (Lapchak et al. 1989). Pharmacological results reveal that along with its specificity and high affinity, [³H]NMCC is selectively displaced by agonists (Araujo et al. 1988; Lapchak et al. 1989), making it a desirable ligand to screen for potential agonistic compounds.

The purpose of the following assay is to determine the binding affinity of potential nicotinic cholinergic agonists in brain, using an agonist ligand.

PROCEDURE

Reagents

- 0.5 M Tris buffer, pH 7.7
 - 57.2 g Tris HCl
16.2 g Tris Base
q.s. to 1 liter with distilled water
 - Make a 1:10 dilution in distilled H₂O (0.05 M Tris buffer, pH 7.7 at 25°C)
- Tris buffer containing physiological ions
 - Stock buffer
NaCl 7.014 g

KCl 0.372 g
 CaCl₂ 0.222 g
 MgCl₂ 0.204 g q.s. to 100 ml with
 0.5 M Tris buffer

b) Dilute 1:10 in distilled H₂O

This yields 0.05 M Tris HCl, pH 7.7, containing NaCl (120 mM), KCl (5 mM), CaCl₂ (2 mM) and MgCl₂ (1 mM).

- Methylcarbamoylcholine iodide, [N-Methyl-³H]- is obtained from New England Nuclear
 For IC₅₀ determinations [³H]-NMCC is made up to a concentration of 100 nM in distilled H₂O and 50 μl added to each tube (yields a final concentration of 5 nM in the 1 ml assay).
- (-)-Nicotine ditartrate is obtained from Research Biochemicals Incorporated.
 A stock solution of (-)-nicotine ditartrate is made up to a concentration of 0.5 mM in distilled H₂O. Twenty μl of stock is added to 3 tubes for the determination of nonspecific binding (yields a final concentration of 10 μM in the assay).
- Test compounds. For most assays in 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2 × 10⁻⁵ to 2 × 10⁻⁸ M. Seven concentrations are used for each assay and higher or lower concentrations may be used depending on the potency of the drug.
- A 0.5% (w/v) solution of polyethyleneimine is prepared in distilled H₂O. GF/B filters are soaked in this solution for at least four hours at 4°C. This is done to reduce binding of the ligand to the filter strips.

Tissue Preparation

Male Wistar rats are decapitated, their frontal cortices removed, weighed and homogenized in 40 volumes of ice-cold 0.05 M Tris buffer, pH 7.7 (1 b). The homogenate is centrifuged at 48,000 g for 10 min. The pellet is rehomogenized in fresh buffer and re-centrifuged at 48000g for 10 min two more times. The final pellet is resuspended in the original volume of buffer, but with physiological salts (2b). This yields a final tissue concentration of 20 mg/ml in the assay.

Assay

- 130 μl 0.05 M Tris pH 7.7-physiological salts (2 b).
 20 μl Vehicle (for total binding) or 0.5 mM (-)-nicotine ditartrate (for nonspecific binding) or appropriate drug concentration.
 50 μl N-[³H]Methylcarbamylcholine stock solution
 800 μl tissue.

The tubes are incubated at 0°C for 60 min. The assay is stopped by rapid filtration through Whatman GF/B filters which are then washed 4 times with 3 ml of ice-cold 0.05 M Tris buffer, pH 7.7. The filters are then counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of the 10 μM (-)-nicotine ditartrate. Specific binding is about 1% of the total added ligand and 60–70% of the total bound ligand. IC₅₀ calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATION OF THE METHOD

Pabreza et al. (1991) recommended [³H]cytisine as a useful ligand for studying neuronal nicotinic receptors because of its high affinity and low nonspecific binding.

Badio and Daly (1994) determined [³H]nicotine receptor binding in rat cerebral cortex membrane preparations. The authors concluded that the analgesic activity of epibatidine, an alkaloid originally characterized from frog skin, is due to its activity as nicotinic agonist.

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Uncompetitive NMDA Receptor Antagonism

PURPOSE AND RATIONALE

The uncompetitive NMDA antagonist memantine with moderate affinity to the (+)MK801-binding site has shown positive effects in the treatment of dementia (Möbius et al. 2004; Sonkusare et al. 2005). Several studies were performed to elucidate the mode of action (Frankiewicz and Parsons 1999; Parsons et al. 1999, 2004; Ikonomidou et al. 2000; Linden et al. 2001; Blanpied et al. 2005; Chen and Lipton 2005; Losi et al. 2006; Volbracht et al. 2006; Zoladz et al. 2006).

Sobolevsky and Koshelev (1998) and Sobolevsky et al. (1998, 1999) studied the blocking effects of memantine in open *N*-methyl-D-aspartate channels.

PROCEDURE

Pyramidal neurons were acutely isolated from the CA-1 region of rat hippocampus using “vibrodissociation techniques” (Vorobjev 1991). The experiments began after 3 h of incubation of the hippocampal slices in a solution containing (in mM): NaCl, 124; KCl, 3; CaCl₂, 1.4; MgCl₂, 2; glucose, 10; and NaHCO₃, 26. The solution was bubbled with carbogen at 32°C. During the whole period of isolation and current recording, nerve cells were washed with a Mg²⁺-free solution containing 3 μ M glycine (in mM: NaCl, 140; KCl, 5; CaCl₂, 2; glucose, 15; and HEPES, 10, pH 7.3). Fast replacement of superfusion solutions was achieved by using the concentration jump technique (Benveniste et al. 1990; Vorobjev 1991) with one application tube. This technique allows substitution of the tubular solution for the flowing solution with a time constant <30 ms but backward with the time constant of 30–100 ms (Sobolevsky 1999). Therefore, except where noted, the rate of the solution exchange was fast at the beginning of any application and slightly slower at its termination. The currents were recorded at 18°C in the whole-cell configuration using micropipettes made from Pyrex tubes and filled with an “intracellular” solution (in mM: CsF, 140; NaCl, 4; and HEPES, 10; pH 7.2). Electrical resistance of the filled micropipettes was 3–7 M Ω . Analog current signals were digitized at 1 kHz frequency.

EVALUATION

Statistical analysis was performed using the scientific and technical graphics computer program Microcal Origin (version 4.1 for Windows). The data presented are mean \pm SE; comparison of the means was done by ANOVA, with $p < 0.05$ taken as significant.

MODIFICATIONS OF THE METHOD

Maskell et al. (2003) reported inhibition of human $\alpha 7$ nicotine acetylcholine receptors by open channel blockers of *N*-methyl-D-aspartate receptors. Human $\alpha 7$ nicotine acetylcholine receptors were expressed in *Xenopus* oocytes and the effects of the NMDA receptor open channel blockers memantine and cerestat on this receptor were examined using two-electrode voltage-clamp recordings and the 125 I-labeled α -bungarotoxin binding.

Aracava et al. (2005) found that memantine blocks $\alpha 7^*$ nicotinic acetylcholine receptors more potently than *N*-methyl-D-aspartate receptors in rat hippocampal neurons. Primary hippocampal cultures were from 16- to 29-day-old fetal rats. Electrophysiological recordings were obtained from cultured neurons by means of the whole-cell mode of the patch-clamp technique. Atropine (1 μ M) and tetrodotoxin (0.1–0.3 μ M) were added to the external solution to block muscarinic receptors and voltage-gated Na^+ channels, respectively. The agonist solutions, with or without memantine, were applied to the neurons through a glass U-tube. Memantine was added at various concentrations to the bathing solution.

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F.2.0.11**Secretion of Nerve Growth Factor by Cultured Neurons/Astroglial Cells****PURPOSE AND RATIONALE**

Cultured brain cells can be used for many purposes such as investigation of synthesis and secretion of nerve growth factor or for testing neuroprotective drugs (Peruche and Kriegstein 1991). Nerve growth factor is required for the development and maintenance

of peripheral and sensory neurons (Thoenen and Barde 1980). Nerve growth factor (NGF) prevents neuronal death after brain injury (Hefti 1986; Williams et al. 1986; Kromer 1987), especially in basal forebrain nuclei involved in memory processes. Drug induced increase in nerve growth factor secretion may be beneficial in primary degenerative dementia.

NGF belongs to the family of neurotrophins which includes besides of the nerve growth factor, the neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5) and brain-derived neurotrophic factor (BDNF). Two types of neurotrophin transmembrane receptors are known: (1) a receptor termed p75, which is common to all neurotrophins and (2) a family of neurotrophin receptor tyrosine kinases *trkA*, *trkB* and *trkC* (Saragovi and Gehring 2000). *TrkA* is the receptor tyrosine kinase for NGF (Huang and Reichardt 2003).

NGF has a crucial role in the generation of pain and hyperalgesia in several acute and chronic pain states (Hefti et al. 2006).

PROCEDURE

Whole brains of 8-day-old mice (ICR) are dissected out and cut into small pieces. The pieces are washed with calcium- and magnesium-free phosphate-buffered saline, treated with 0.25% trypsin at 37°C for 30 min, and triturated with a Pasteur pipette. The excess trypsin is removed by centrifugation at 200 g for 5 min. The cells or cell clumps from one brain are cultured in a culture bottle with Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS), 50 milliunits/ml penicillin, and 50 mg/ml of streptomycin at 37°C in a humid atmosphere of 5% CO₂ for 1–2 weeks with medium changes every 3 days. After confluence is reached, the cells in each bottle are dissociated by trypsin treatment and recultured in new bottles. This procedure is repeated 3 times. The culture becomes composed of morphologically uniform cells.

Preparation of quiescent cells is performed by inoculating into 96-well plates and culture in FCS-containing DMEM until confluence is reached. Then, the cells are cultured for an additional week in FCS-free DMEM containing 0.5% BSA, with medium changes every 3 days. Because the cells never proliferate in FCS-free medium, most of the cells are arrested in the quiescent phase. Then, the medium is changed to DMEM containing 0.5% BSA with or without drugs, and the cells are cultured for 24 h.

Nerve growth factor (NGF) content in the culture medium is determined by a two-site enzyme immunoassay (Furukawa et al. 1983; Lärkfors and Eben-

dal 1987). Mouse β NGF isolated from male mouse submaxillary glands is purified by CM-Sephadex C-50 chromatography. Antiserum to this mouse β NGF is produced in New Zealand White rabbits by repeated subcutaneous injections of an emulsion in complete Freund's adjuvant over 18 months. Immunoglobulin G is prepared from Anti-mouse β NGF antiserum by Sepharose chromatography. Antibody IgG is incubated with pepsin and chromatographed. Fab' fragments are coupled to β -D-galactosidase. IgG-coated solid phase is prepared in polystyrene tubes. The IgG-coated polystyrene tubes are incubated with 0.25 ml buffer containing various amounts of NGF with gentle shaking. After incubation for 18–24 h at 4°C, each tube is washed twice with 1 ml of buffer and 0.13 milliunits of the Fab'- β -D-galactosidase complex in 0.25 ml buffer is added. After incubation for 18–24 h at 4°C with gentle shaking each tube is washed as described above and β -D-galactosidase activity bound to the tube is assayed. The enzyme reaction is started by addition of 60 mM 4-methylumbelliferyl- β -D-galactoside and 0.1% Triton X-100 in 0.25 ml buffer. After 1-h incubation at room temperature, the enzyme reaction is stopped by the addition of 1.25 ml 0.1 M glycine-NaOH buffer (pH 10.3). The amounts of 4-methylumbelliferone formed are measured by fluorometry (Excitation wavelength 360 nm, emission wavelength 450 nm).

EVALUATION

Time-response curves of release of NGF into the medium are established after addition of drug and compared with controls. Dose-response curves can be prepared after addition of various amounts of test drug.

MODIFICATIONS OF THE METHOD

Cultured neurons from chick embryo hemispheres were used for testing cerebroprotective drug effects *in vitro* and for testing antihypoxic drug effects by Krieglstein et al. (1988), Peruche et al. (1990), Oberpichler-Schwenk and Krieglstein (1994).

Semkova et al. (1996) found that clenbuterol protects mouse cerebral cortex and rat hippocampus from ischemic damage and attenuates glutamate neurotoxicity in cultured hippocampal neurons by induction of nerve growth factor.

Prehn et al. (1993, 1995) tested the prevention of glutamate neurotoxicity in neocortical cultures from rats. Mixed neuronal/glial primary cultures were derived from the cerebral cortices of neonatal Fischer 344 rats. Excitotoxic injury was induced after 14 days

by L-glutamate following a procedure described by Choi et al. (1988), Koh and Choi (1988).

Kinoshita et al. (1991) used primary cultured neurons from 17-day-old rat fetuses.

Qi et al. (1997), Horton et al. (2001) described a novel catecholaminergic CAD CNS neuronal cell line in which neurotrophin-3 mediates the autocrine survival.

Shinpo et al. (1999) used cultured mesencephalic neurons from embryonic Sprague Dawley rats to study the protective effects of the TNF-ceramide pathway against glutamate neurotoxicity.

Matsumoto et al. (1990) described a method for quantifying the effects of neurotrophic factors on the number of surviving neurons and the total length of neurites in primary cultures from cerebral cortex and hippocampus of the brains from two-week old rats by using digital image processing techniques. Binary images of neuronal neurites were extracted from gray images of cultured neurons stained with Coomassie brilliant blue.

White et al. (1995) measured calcium transients in mouse cerebellar granule cells with the Ca^{2+} -sensitive probe indo-1/AM.

Beresini et al. (1997) developed two types of high throughput assays to identify small molecules that interact with neurotrophin receptors. The first, the receptor binding assay, is a competitive binding assay that uses a recombinant receptor fusion protein and biotinylated neurotrophin. This assay detects compounds that inhibit neurotrophin binding to the receptor; these compounds may be either agonistic or antagonistic. The second assay, the kinase receptor activation ELISA, detects receptor autophosphorylation in response to sample- or neurotrophin-stimulation of receptor transfected cells. Receptor autophosphorylation is evaluated by analyzing lysates of the stimulated cells in a receptor-specific ELISA for phosphotyrosine residues. This assay is bioactivity based, and consequently, has the power of detecting as well as distinguishing receptor agonists and antagonists.

Höglinger et al. (1998) used free-floating roller tube cultures prepared from embryonic-day-14 rat ventral mesencephalon to study the influence of brain-derived neurotrophic factor treatment on dopamine neuron survival and function.

Nerve growth factor is crucial for survival of nociceptive neurons during development. Shu and Mendell (1999) investigated the acute effects of NGF on capsaicin responses of small-diameter dorsal root ganglion cells in culture.

For further studies with brain cell cultures see E.3.1.16.

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F.2.0.12

Inhibition of Respiratory Burst in Microglial Cells/Macrophages

PURPOSE AND RATIONALE

Activated phagocytes can produce large amounts of oxygen intermediates that result from a process during which NADPH oxidase reduces O_2 to the superoxide anion (O_2^-), which subsequently dismutates together with H^+ to H_2O_2 and O_2 . H_2O_2 is then reduced to hypochlorous acid by myeloperoxidase (Bellavite 1988). The cascade of metabolic steps is known as respiratory bursts. The intracellular formation of reactive oxygen intermediates can be determined by measuring the oxidation of the membrane permeable and non-fluorescent dihydrorhodamine 123 to the cationic and intracellularly trapped, green fluorescent rhodamine 123 in single viable cells. Microglial/brain macrophage-mediated damage in the central nervous system is accompanied by an increased production of free radicals, which also seems important in primary degenerative dementia (Alzheimer's disease) (Banati et al. (1993). Inhibition of this process by drugs may indicate therapeutic value in Alzheimer's disease.

PROCEDURE

Cell Culture

Cultures of new-born rat brain are prepared as described by Guilian and Baker (1986); Frei et al. (1987). Isolated cerebral cortices from new-born albino rats are stripped of the meninges, minced in culture medium and dissociated by trituration for 2 h in 0.25% trypsin solution. Cells are plated in 75 cm^2 plastic culture flasks containing 10 ml medium with 10% fetal bovine serum at a density of 85,000 cells/ml. After 7 days, confluent cultures are vigorously agitated on a rotary shaker at 37°C for 15 h. Glial fibrillary acid protein positive astroglia remain adherent to the flasks. The resulting cell suspension, rich in amoeboid microglia and oligodendroglia, is placed in plastic flasks and allowed to adhere at 37°C. After a 1–3 h adhering interval, loosely adhering and suspended cells (most of which are oligodendroglia) are removed by gently shaking the flasks at room temperature. The strongly adherent microglia cells are then released by vigorous shaking in medium with 0.2% trypsin. Once the majority of microglia is suspended, fetal bovine serum is added (15% final volume), and the cell suspension added to new flasks. After a second 1–3 h interval to allow adhesion, the medium is removed, and adhering microglia are suspended using trypsin. Final prepara-

tion show a nearly homogeneous population of non-specific esterase positive cells.

Peritoneal macrophages are obtained from 12-week-old male Wistar rats.

For flow cytometric measurement, cells are suspended ($3\text{--}4 \times 10^6$ cells/ml) in Hank's buffered saline (HBS; Sigma Chemie, Deisenhofen, Germany) supplemented with N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES; 5 mM, pH 7.35; Serva Feinbiochemica, Heidelberg, Germany) (HBS-HEPES) and stored at 4°C for a maximum of 2 h.

Flow Cytometric Measurement of Respiratory Burst

Dihydrorhodamine 123 (DHR) is obtained from Molecular Probes (Eugene, OR, USA) and dissolved to obtain a 1 mM stock solution in N,N-dimethylformamide (DMF; Merck, Darmstadt, Germany).

The cellular suspensions of peritoneal macrophages and microglial cells (10 μ l) are each further diluted with 1 ml HBS-HEPES and stained for 5 min at 37°C with 10 μ l of a 100 μ M DHR solution in HBS (1 mM stock solution in DMF). The DHR-loaded cells are incubated with the test drug at various concentrations for 15, 25, 35, 45, and 60 min with and without Con A (100 μ g/ml; Sigma Chemie) stimulation. The DNA of dead cells is counter stained with 10 μ l of 3 mM propidium iodide (Serva Feinbiochemica) solution in HBS 3 min before the flow cytometric measurement. To exclude effects from a possible release of endogenous adenosine, control experiments with incubation medium containing adenosine deaminase (200 U/mg, 5 μ g/ml Sigma Chemie) are performed.

The forward scatter, side scatter and two fluorescences of at least 10,000 cells/sample are measured simultaneously on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Rhodamine 123 green fluorescence (515–545 nm) and propidium iodide red fluorescence (>650 nm) are measured with the light from an argon laser of 488-nm excitation wavelength.

EVALUATION

The differences in respiratory burst activities caused by Con A stimulation and treatment with test drug are tested for significance by unpaired *t*-test. The *t*-test is performed for each time point including the data of at least four independent experiments. Each single fluorescence value of each experiment is based on the measurement of at least 10,000 cells. Before each experiment the flow cytometer is calibrated with standardized yellow-green fluorescent microspheres of 4.3- μ m diameter (Polysciences, St. Goar, Germany),

thus ensuring the compatibility of the fluorescence values from different experimental series.

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F.3 In Vivo Methods

F.3.1 Inhibitory (Passive) Avoidance

One of the most common animal tests in memory research is the inhibition to imitate activities or learned habits. The term “passive avoidance” is usually employed to describe experiments in which the animal learns to avoid a noxious event by suppressing a particular behavior. Netto and Izquierdo (1985) have discussed this term in a brief paper. The term “inhibitory avoidance” is used more often in later publications.

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F.3.1.1 Step-down

PURPOSE AND RATIONALE

An animal (mouse or rat) in an open field spends most of the time close to the walls and in the corners. When placed on an elevated platform in the center of a rectangular compartment, it steps down almost immediately to the floor to explore the enclosure and to approach the wall. The technique is employed in different mod-

ifications, which are described by Jarvik and Essmann (1960), Hudspeht et al. (1964), Chorover and Schiller (1965), Kubanis and Zornetzer (1981), Zornetzer et al. (1982), Abdel-Hafez et al. 1998).

PROCEDURE

Mice or rats of either sex are used. A rectangular box (50 × 50 cm) with electrifiable grid floor and 35 cm fits over the block. The grid floor is connected to a shock device, which delivers scrambled foot shocks. The actual experiments can be performed in different ways. A typical paradigm consists of three phases: (1) Familiarization: The animal is placed on the platform, released after raising the cylinder, and the latency to descend is measured. After 10 s of exploration, it is returned to the home cage. (2) Learning: Immediately after the animal has descended from the platform an unavoidable footshock is applied (Footshock: 50 Hz; 1.5 mA; 1 s) and the animal is returned to the home cage. (3) Retention Test: 24 h after the learning trial the animal is again placed on the platform and the step-down latency is measured. The test is finished when the animal steps down or remains on the platform (cut-off time: 60 s).

EVALUATION

The time of descent during the learning phase and the time during the retention test is measured. A prolongation of the step-down latency is defined as learning.

MODIFICATIONS OF THE TEST

This test procedure is employed in different modifications. One of the most common modifications is to induce amnesia in animals. There are different methods to do so including (i) electroconvulsive shock, (ii) scopolamine, (iii) alcohol, (iv) CO₂.

Ricceri et al. (1996) studied the effect of nerve growth factor on passive avoidance learning and retention in developing mice. At the beginning of each trial 11- or 15-day-old pups were placed on the central platform. Immediately thereafter, the platform began vibrating and a timer was simultaneously activated. A trial was ended when the pup gave a step-down response, or after 120 s without descent from the platform. Each step-down response was followed by a 3-s 0.3-mA foot shock.

CRITICAL ASSESSMENT OF THE METHOD

The variability of this method is relative high; therefore, it is necessary to test large groups of animals (minimum 10 animals per group). There are some critical parts in the experimental procedure: (i) Placing the

animal on the platform, since the tendency of the animal to escape the contact with the human hand may shorten the step-down latencies. (ii) Another important point is the timing of the electric shock. It must not be applied at the first contact of the animal with the floor, since the light touch with the forelimbs does not cause the required shock intensity. The duration and intensity of the shock should be constant. (iii) It is also necessary to keep the room sound-proof, and this can be done by using a white noise generator (60–70 dB). Due to all these critical variables the results of different authors are difficult to compare.

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F.3.1.2 Step-through

PURPOSE AND RATIONALE

This test uses normal behavior of mice and rats. These animals avoid bright light and prefer dim illumination. When placed into a brightly illuminated space connected to a dark enclosure, they rapidly enter the dark compartment and remain there. The standard technique was developed for mice by Jarvik and Kopp (1967) and modified for rats by King and Glasser (1970). It is widely used in testing the effects of mem-

ory active compounds (Fekete and de Wied 1982; Hock and McGaugh 1985; Hock et al. 1989; Hock 1994).

PROCEDURE

Mice and rats of either sex are used. The test apparatus consists of a small chamber connected to a larger dark chamber via a guillotine door. The small chamber is illuminated with a 7 W/12 V bulb. The test animals are given an acquisition trial followed by a retention trial 24 h later. In the acquisition trial the animal is placed in the illuminated compartment at a maximal distance from the guillotine door, and the latency to enter the dark compartment is measured. Animals that do not step through the door within a cut-off time: 90 s (mice) or 180 s (rats) are not used. Immediately after the animal enters the dark compartment, the door is shut automatically and an unavoidable footshock (Footshock: 1 mA; 1 s – mice; 1.5 mA; 2 s – rat) is delivered. The animal is then quickly removed (within 10 s) from the apparatus and put back into its home cage. The test procedure is repeated with or without drug. The cut-off time on day 2 is 300 s (mice) or 600 s (rats), respectively.

EVALUATION

The time to step-through during the learning phase is measured and the time during the retention test is measured. In this test a prolongation of the step-through latencies is specific to the experimental situation. An increase of the step-through latency is defined as learning.

CRITICAL ASSESSMENT OF THE METHOD

Same as in step-down method

MODIFICATIONS OF THE TEST

This test procedure is employed in different modifications. To test drugs usually several test-groups can be tested. (i) CXC-group: vehicle – without amnesia – vehicle; (ii) DXD-group: drug – without amnesia – drug; (iii) CAC-group: vehicle – amnesia – vehicle; (iv) DAD-group: drug – amnesia – drug; (v) CAD-group: vehicle – amnesia – drug; (vi) DAC-group: drug – amnesia – vehicle. (The first figure means treatment on day 1; the third figure means treatment on day 2). There are different methods to induce amnesia: (i) electroconvulsive shock, (ii) scopolamine, (iii) alcohol, (iv) CO₂ etc.

Step-through experiments were performed after unilateral ibotenic acid lesions in the right nucleus basalis magnacellularis as a rat model of Alzheimer disease by Fine et al. (1985).

Wan et al. (1990) recorded changes in heart rate and body temperature via a telemetry system during step through passive avoidance behavior in rats.

Picciotto et al. (1995) used gene targeting to mutate β_2 , the most widely expressed neuronal nicotinic acetylcholine receptor in the central nervous system for pharmacological and behavioral studies. Retention of the inhibitory avoidance response was better in mutant mice than in their non-mutant siblings.

Galeotti et al. (1997) modified the step-through method. After entry to the dark compartment, mice received a non-painful punishment consisting of a fall into a cold water bath (10°C). For this purpose the dark chamber was constructed with a pitfall floor. The memory degree of received punishment (fall into cold water) was expressed as the increase (in seconds) in the retention latency in comparison with the training latency.

Itokazu et al. (2001) showed that L-DOPA caused memory deficits in mice in a step-through passive avoidance test and recommended this as a model for human dementia.

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F.3.1.3

Two Compartment Test

PURPOSE AND RATIONALE

A rodent in an open field tends to enter any recesses in the walls and to hide there. When placed into a large box, connected through a narrow opening with a small dark compartment, the animal rapidly finds the entrance into the small chamber, enters it and spends most of its time there. The times spent in the large and small compartments are measured. The latency of the first entrance into the dark chamber and the number of crossings from one compartment into the other can be used as auxiliary criteria. The technique described was developed by Kurtz and Pearl (1960) and modified by Bures and Buresova (1963).

PROCEDURE

Mice and rats of either sex and a rectangular box with a 50 × 50 cm grid floor and 35 cm high walls are used. In the centre of one wall is a 6 × 6 cm opening connecting the large compartment to a small 15 × 15 cm box with dark walls, electrifiable grid floor and removable ceiling. The connection between the two compartments can be closed with a transparent sliding door. Illumination is provided with a 100 W bulb placed 150 cm above the centre of the large compartment.

EVALUATION

The times the animal spends in the large and the small compartment are measured.

CRITICAL ASSESSMENT OF THE METHOD

Same as in step-down method.

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F.3.1.4

Up-Hill Avoidance

PURPOSE AND RATIONALE

Many animal species exhibit a negative geotaxis, i. e. the tendency to orient and move towards the top when placed on a slanted surface. When placed on a tilted platform with head facing down-hill, rats and mice invariably turn around and move rapidly up the incline (Staubli and Huston 1978).

PROCEDURE

Rats of both sex were used and maintained under standard conditions. The experimental apparatus is a 50 × 50 cm box with 35 cm high opaque plastic walls. The box can be inclined at different angles. The floor consists of 10 mm diameter stainless steel grid bars placed 13 mm apart. To deliver the tail-shock, a tail-electrode is constructed, consisting of a wire clip connected to a constant current shock source. The animal is first fitted with the tail-electrode and then placed onto the grid with its nose facing down. During baseline-trials the animal's latency to make a 180° turn and initiate the first climbing response is measured. Thereafter the animal is returned to its home cage. During the experimental trials the latencies are measured and additionally a tail-shock (1.5 or 2 mA) was administered contingent on the first climbing response after the 180° turn. Immediately after the shock the animal is placed in its home cage. Retest is performed 24 h later.

EVALUATION

The latencies are measured.

CRITICAL ASSESSMENT OF THE METHOD

The up-hill avoidance technique promises to provide a useful addition to the existing arsenal of inhibitory (passive) avoidance methods. Its most obvious advantage is that it can be administered to animals debilitated in sensory-motor coordination by pharmacological or surgical treatments that would preclude use of other inhibitory (passive) avoidance tasks.

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F.3.1.5**Trial-to-criteria inhibitory avoidance****PURPOSE AND RATIONALE**

As animals experience different sensitivity to the footshock punishment applied in the dark area, immediately after the first trial the animal is returned to the lighted area to evaluate if the task has been acquired. A criteria is established to determine the learning of the test, usually requiring the animal to remain in the lighted area for a period of 30–60 s. (Decker et al. 1990). In this way, all the animals have a similar degree of learning independently of the amount of trials needed to attain it.

PROCEDURE

Mice or rats are generally used. The animals are trained in the same way as in the step-through version. They are placed in the lighted compartment and after they entered with the four paws into the dark area, the door is closed and a mild footshock is delivered. Immediately after the shock they are placed back in the lighted area for another trial. Training would continue this way until the animal remains in the lighted area for a certain period of time (30 or 60 s), a time at which the training is considered to be acquired by all the animals. The number of trials to attain criteria are counted as an indication of the speed of acquisition.

EVALUATION

Retention of the test is measured 24 or 48 Hs later. The animals are placed in the lighted area, the door opened and the latency to step with the four paws into the dark area is recorded. A cut-off latency of 180 or 300 s is usually imposed.

CRITICAL ASSESSMENT OF THE TEST

This modification of the classical inhibitory avoidance procedure is useful to determine the effect of drugs on acquisition as amnesic drugs significantly increase the number of trials to reach the criterion. Saline-treated animals generally need 1–2 trials to reach criterion while animals under diazepam or scopolamine need 3–6 trials. Once the animals have been trained to a predetermined criteria (i. e., 30 s. avoidance) any effect of the drug on the retention trial can be associated to drug effects on consolidation rather on the acquisition process (Brioni 1993; Decker et al. 1990).

Alternatively, drugs could be administered before the test session in order to determine the effect of the drug on retrieval processes. Brain lesions differentially affect acquisition or retention (Hepler et al. 1985; Tomaz et al. 1992), and this task has also been used to demonstrate the effect of grafts on recovery of function (Fine et al. 1985).

MODIFICATIONS OF THE TEST

This test can also be performed as a continuous trial-to-criterion. In this procedure the door is not closed after the animal enters the dark area and upon delivery of the shock the animal can escape to the lighted area. The experimenter does not have to touch the animal to place it in the lighted area for the next trial.

Kameyama et al. (1986) described a similar model for the study of learning and memory in mice using a step-down-type passive avoidance- and escape-learning method.

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F.3.1.6**Scopolamine-Induced Amnesia in Mice****PURPOSE AND RATIONALE**

The administration of the antimuscarinic agent scopolamine to young human volunteers produces transient memory deficits (Drachman and Leavitt 1974). Analogously, scopolamine has been shown to impair memory retention when given to mice shortly before training in a dark avoidance task (Dilts and Berry 1967; Glick and Zimmerberg 1972; Schindler et al. 1984). The ability of a range of different cholinergic agonist drugs to reverse the amnesic effects of scopolamine is now well documented in animals and human volun-

teers. However, the neuropathology of dementia of the Alzheimer type is not confined to the cholinergic system (Iversen 1998).

PROCEDURE

The scopolamine test is performed in groups of 10 male NMRI mice weighing 26–32 g in a one-trial, passive avoidance paradigm. Five min after i.p. administration of 3 mg/kg scopolamine hydrobromide, each mouse is individually placed in the bright part of a two-chambered apparatus for training. After a brief orientation period, the mouse enters the second, darker chamber. Once inside the second chamber, the door is closed which prevents the mouse from escaping, and a 1 mA, 1-s foot shock is applied through the grid floor. The mouse is then returned to the home cage. Twenty-four hours later, testing is performed by placing the animal again in the bright chamber. The latency in entering the second darker chamber within a 5 min test session is measured electronically. Whereas untreated control animals enter the darker chamber in the second trial with a latency of about 250 s, treatment with scopolamine reduces the latency to 50 s. The test compounds are administered 90 min before training. A prolonged latency indicates that the animal remembers that it has been punished and, therefore, does avoid the darker chamber.

EVALUATION

Using various doses latencies after treatment with test compounds are expressed as percentage of latencies in mice treated with scopolamine only. In some cases, straight dose-response curves can be established whereas with other drugs inverse U-shaped dose-responses are observed (Schindler et al. 1984).

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that the pathogenesis of primary degenerative dementia (Alzheimer's disease) in man has been only partially elucidated, the scopolamine-amnesia test is widely used as primary screening test for so-called anti-Alzheimer drugs.

MODIFICATIONS OF THE METHOD

Lenègre et al. (1988) investigated the effects of piracetam on amnesias induced by scopolamine, diazepam and electroconvulsive shock.

Yamaoto et al. (1990) studied the effect of drugs on the impairment of working memory produced by scopolamine, ethylcholine aziridinium (AF64A) or cerebral ischemia using a repeated acquisition procedure in a three-panel runway apparatus.

Hiramatsu and Inoue (2000) demonstrated improvement by low doses of nociceptin on scopolamine-induced impairment of learning and/or memory in mice.

Martin et al. (1995) studied reversal of scopolamine-induced memory impairment by aniracetam in **rats** in an experiment conducted over a 1-year period.

Braida et al. (1998) investigated the short (120 min) and long-lasting (360 min) antagonism of scopolamine-induced amnesia in rats in an eight-arm radial maze by a cholinesterase inhibitor.

Giovannini et al. (1999) investigated the effects of histamine H₃ receptor agonists and antagonists on cognitive performance and scopolamine-induced amnesia.

Several **other drugs** have been shown to induce memory impairment.

Amnesia can also be induced by pretreatment with **benzodiazepines** (Porsolt et al. 1988).

Nabeshima et al. (1986) studied the antagonism against **phencyclidine**-induced retrograde amnesia in mice.

Maurice and Privat (1997) examined learning impairment induced by the non-competitive NMDA receptor agonist **dizocilpine** in mice.

Pitts et al. (2006) showed that **chlordiazepoxide** and **dizocilpine**, but not morphine, selectively impair acquisition under a novel repeated-acquisition and performance task in rats.

Ellison (1995) reported that the **N-methyl-D-aspartate antagonists** phencyclidine, ketamine, and dizocilpine provide behavioral and anatomical models of dementia.

Tanaka et al. (1998) studied learning and memory deficits induced by **β -amyloid** protein in rats.

Anandatheerthavarada et al. (2003) reported that mitochondrial targeting and a transmembrane arrest of Alzheimer's **amyloid precursor protein** impairs mitochondrial function in neuronal cells.

Ukai et al. (2001) showed that **endorphins** 1 and 2, endogenous μ -opioid receptor agonists, impair passive avoidance learning in rats.

Patil et al. (2002) used **lipopolysaccharide**-intoxicated mice as a model for Alzheimer's disease.

Yang et al. (2004) induced learning and memory impairment in mice by treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**MPTP**).

Sparks and Schreurs (2003) reported that trace amounts of **copper** in water induce β -amyloid plaques and learning deficits in a **rabbit model** of Alzheimer's disease using the nictitating membrane response (Buck et al. 2001).

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F.3.1.7

Cognitive Deficits on Chronic Low Dose MPTP-Treated Monkeys

PURPOSE AND RATIONALE

Röltgen and Schneider (1994), Schneider et al. (1990, 1993, 1994a, b) described the effect of chronic low-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure on cognitive functions in monkeys. These animals develop cognitive deficits and difficulties in performing previously learned tasks, such as delayed response, delayed matching-to-sample, delayed alternation, visual discrimination reversal, and object retrieval, as well as dopamine depletions in several brain areas without severe Parkinsonian signs as found after high MPZP doses (Burns et al. 1983), see E.7.1.2. In addition, these animals exhibit other behavioral changes such as decreased task persistence, increased restlessness and decreased attention.

PROCEDURE

Adult Macaca monkeys are trained to perform a delayed response task while seated in a restraining chair placed inside a sound attenuating modified Wisconsin General Test apparatus. The monkey sits behind an opaque screen that when raised allows access to a sliding tray that contains recessed food wells with identical sliding white Plexiglas covers that serve as stimulus plaques that can be displaced by the animal to obtain rewards (raisins). Monkeys are trained to retrieve a raisin from one of the food wells after observing the experimenter baits the well. Right and left wells are baited in a randomized, balanced order. Animals are maintained on a restricted diet during the week and tested while food deprived. Training is accomplished with a non-correction procedure with 0 s delay and progressing to 5 s delay. Animals are trained until performance with a 5 s delay is 90% correct or

better for at least 5 consecutive days. Each daily session consists of 25 trials. A response is scored a 'mistake' if the monkey makes its response choice to a well that is not baited with reward. A 'no response' error is scored if the monkey fails to respond to a trail within 30 s.

Once the animals are performing at criterion level, MPTP is administered intravenously in doses ranging from 0.05 mg/kg at the start of the study to 0.20 mg/kg. Animals receive cumulative doses up to 60 mg over periods up to one year.

Pharmacological data are obtained after animals consistently show at least a 15% performance deficit on delayed response. Drugs are administered subcutaneously. Delayed response testing begins 8 min after drug administration. On drug testing days, animals receive drug or saline and are tested for delayed response performance, administered drug (or saline), and retested on the delayed response task.

EVALUATION

Delayed response performance after drug administration is compared with matched control performance obtained on the same day prior to drug administration. The total number of correct responses as well as the number of mistakes and 'no response' errors are tabulated for each session. Data are then expressed as mean (\pm standard deviation) performance. All animals serve as their own controls. Statistical analysis consists of analysis of variance, repeated measures design, with post hoc comparisons (Bonferroni *t*-test).

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F.3.2

Active Avoidance

Active avoidance learning is a fundamental behavioral phenomenon (Herrnstein 1969; Brush 1971; Campbell and Church 1969; D'Amato 1970). As in other instrumental conditioning paradigms the animal learns to control the administration of the unconditioned stimulus by appropriate reactions to the conditioned stimulus preceding the noxious stimulus. The first stage of avoidance learning is usually escape, whereby a reaction terminates the unconditioned stimulus.

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F.3.2.1

Runway Avoidance

PURPOSE AND RATIONALE

A straightforward avoidance situation features a fixed aversive gradient which can be traversed by the animal. The shock can be avoided when the safe area is reached within the time allocated (Munn 1950; Capaldi and Capaldi 1972; Hock and McGaugh 1985).

PROCEDURE

Mice or rats of either sex are used and maintained under standard conditions and handled for several days before the experiment. The same box as used in the step-through model can be used in this experiment. The apparatus is uniformly illuminated by an overhead light source. A loudspeaker, mounted 50 cm above the start-box, serves for presenting the acoustic conditioned stimulus (CS; an 80 dB, 2000 Hz tone from an audiogenerator). The footshock is employed by the same source as in the step-through avoidance. The animal is allowed to explore the whole apparatus for 5 min. The guillotine door is then closed and the animal is placed into the light starting area. After 10 s the acoustic CS is applied and the door is simultaneously opened. Shock is turned on after 5 s. The CS continues until the animal reaches the safe area. It is left there for 50–70 s (intertrial interval, ITI) before returned to the same area again. The procedure starts again. The training is continued until the animal attains the criterion of 9 avoidances in 10 consecutive trials. On the

next day the procedure is repeated until the same learning criterion is reached. The time needed to reach the safe area is measured.

EVALUATION

The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) is recorded.

MODIFICATIONS OF THE TEST

Several modifications are described in the literature. The CS also can be a light signal. The number of trials and the criterion can be changed.

CRITICAL ASSESSMENT OF THE METHOD

Same as in step-down method.

REFERENCES AND FURTHER READING

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the connecting door open and the compartment lights switched off. The guillotine door is then closed. After 20 s the light is switched on in the compartment containing the animal, and the door is opened. A tone (CS) is presented and 5 s later the floor shock is applied in the illuminated compartment and continued until the animal escapes to the dark side of the compartment, the connecting door is closed and the shock discontinued. After a variable intertrial interval (ITI; 30–90 s) the light is switched on in the previous dark compartment, the door is opened and the animal is required to cross to the other side. The training is continued until the animal reaches the criterion of 9 avoidances in 10 consecutive trials. Retention is tested at different intervals after the original training by retraining the animal to the same criterion again.

EVALUATION

The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) are recorded.

CRITICAL ASSESSMENT OF THE METHOD

The task is rather difficult due to the lack of a permanent safe area, lack of a simple instrumental response, presence of a variable aversive gradient and increased weight of emotional factor.

MODIFICATIONS OF THE METHOD

Salmi et al. (1994) described a computer-assisted two-way avoidance conditioning equipment for rats.

REFERENCES AND FURTHER READING

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F.3.2.2

Shuttle Box Avoidance (Two-Way Shuttle Box)

PURPOSE AND RATIONALE

Compared to runway avoidance, shuttle box avoidance (two-way-shuttle-box) is a more difficult task. Since the animal is not handled between trials, the shuttle-box can be easily automated (Capaldi and Capaldi 1972).

PROCEDURE

Rats of both sex are used and maintained under standard conditions. The apparatus used consists of a rectangular box 50 × 15 cm with 40 cm high metal walls, and an electrifiable grid floor. The box is divided by a wall with a manually or solenoid-operated guillotine door (10 × 10 cm). into two 25 × 15 cm compartments. Each compartment can be illuminated by a 20 W bulb mounted in the hinged Plexiglas lids. A fixed resistance shock source with an automatic switch (0.5 s on 1.5 s off) is used. Simple programming equipment provides for automatic delivery of the conditioned stimulus (CS) and the unconditioned stimulus (US). The apparatus is placed in a dimly lit room with a masking noise background (white noise) of 60 dB. The animal is allowed to explore the apparatus for 5 min with

F.3.2.3

Jumping Avoidance (One-Way Shuttle Box)

PURPOSE AND RATIONALE

Since a high degree of automation and minimum handling are additional requirements for this model, the obvious solution is a simplified one-way avoidance, allowing for the spontaneous or forced return of the animal to the start. In order to enhance the start-goal

distinction a vertical gradient is introduced which requires the animal to perform a discrete response of an all-or-none character, such as the jump, which clearly differs from the more continuous translational movements required in the usual avoidance tasks (Tenen 1966; McKean and Pearl 1968).

PROCEDURE

Rats of both sex are used and maintained under standard conditions. The apparatus used consists of a rectangular box 40 × 25 cm with 40 cm high metal walls, an electrifiable grid floor and a Plexiglas ceiling. A 12 × 12 × 25 cm opaque plastic pedestal, mounted onto one of the narrow walls of the box provides the isolated goal area. Flush with the horizontal surface of the pedestal moves a vertical barrier, which can either be retracted to the rear wall of the apparatus to expose the goal area or pushed forward to block access to the goal completely. The animal is placed into the apparatus for 5 min with the goal area exposed (barrier retracted). The barrier is then moved forwards and the goal is blocked for 2 s. The first trial starts by exposing the goal area and applying an acoustic CS (1000 Hz, 85 dB). Electric shocks – US (1.0 mA; 50 Hz; 0.5 s) – are applied 5 s later (once per 2 s), and continued together with the CS until the animal jumps onto the platform. After 30 s the barrier pushes the animal off the platform onto the grid floor. The sequence is repeated until the criterion of 10 consecutive avoidances is reached. Retention is tested on the next day until the animal reaches criterion.

EVALUATION

The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) is recorded.

CRITICAL ASSESSMENT OF THE METHOD

In automated procedures extinction is more rapid, especially when short inter test intervals are used.

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F.3.3

Discrimination Learning

In the experiments described above the animals have no choice between the conditioned stimuli. They have only one conditioned stimulus. The following examples illustrate the special techniques employed for dis-

crimination among different stimulus modalities. The experiments can be classified either as simultaneous or successive discrimination paradigms. An exhaustive survey of discrimination studies can be found in Gilbert and Sutherland (1969).

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F.3.3.1

Spatial Habituation Learning²

PURPOSE AND RATIONALE

The open-field test utilizes the natural tendency of rodents to explore novel environments in order to open up new nutrition, reproduction and lodging resources (Birke and Archer 1983; Cerbone and Sadile 1994). The rate of exploratory behaviors exhibited in an unfamiliar environment is limited through the inherent necessity to avoid potential dangers. The observed behavior therefore is always a compromise between these conflicting interests and is regulated in part by the momentary physiological needs (Gerlai 1999; Groves and Thompson 1970). Spatial habituation learning is defined as a decrement in reactivity to a novel environment after repeated exposure to that now familiar environment. This reduction in exploratory behaviors during re-exposures is interpreted in terms of remembering or recognition of the specific physical characteristics of the environment. The test can be used to examine short-term spatial memory (within-trial reductions in exploratory activity) and/or long-term spatial memory (between-trial reductions in exploratory activity after a retention interval of 24, 48 or 96 h after the initial exposure) (Lát 1973).

PROCEDURE

The open-field apparatus is a rectangular chamber (rats: 60 × 60 × 40 cm, mice: 26 × 26 × 40) made of painted wood or grey PVC. A 25 W red or green

²Contribution by E. Dere.

light bulb is placed either directly above or beneath the maze to achieve an illumination density at the centre of approximately 0.3 lx. Masking noise is provided by a broad spectrum noise generator (60 dB). Prior to each trial, the apparatus is swept out with water containing 0.1% acetic acid. Housing room and the testing location are separated and animals are transported to the testing room 30 min before testing. The digitized image of the path taken by each animal is stored and analyzed post hoc with a semi-automated analysis system (e. g. Ethovision, Noldus, The Netherlands or Truscan, Coulbourn Instruments, Allentown, PA). In aged or hypoactive rodents testing is performed during the animals dark phase of day. The rodent is placed on the center or in a corner of the open-field for 5–10 minute sessions (mice: up to 20 min, because of the high basal activity level). The animals are re-exposed to the open-field 24 and 96 h after the initial trial (Thiel et al. 1999; Schwarting et al. 1999).

EVALUATION

The exploratory behaviours registered are: (1) Rearings or vertical activity: the number of times an animal was standing on its hind legs with forelegs in the air or against the wall. (2) The duration of single rearings as a measure of non-selective attention (Aspide et al. 2000) (3) Locomotion or horizontal activity: the distance in centimeters an animal moved.

MODIFICATIONS OF THE TEST

In order to assess emotionality parameters either in interaction with spatial novelty (a single exposure) or in interaction with spatial habituation learning (repeated exposures), the aversivity of the open-field can be varied by increasing the size (inducing agoraphobia) and/or the illumination density (inhibits exploration) of the apparatus (File 1980). Rodents exposed to a big sized brightly lit open field tend to spent more time in the corners or close to the wall and avoid the central part of the apparatus. The emotional behaviors registered are: (1) Corner time: the time spent in the 4 corner squares (rats: 15 × 15 cm; mice: 6.5 × 6.5 cm). (2) Wall time: the time an animal spent close to the wall as a measure for thigmotaxis (scanning the walls of the apparatus with the vibrissae). (3) Center time: the time spent in the center of the open field (rats: 20 × 20 cm; mice: 10 × 10 cm). (4) Defecation: number of boli deposited. (5) Freezing: the time the animal stays completely immobile except for movements associated with respiratory activity (Hall 1936; Frisch et al. 2000). To assess habituation learning not only by decrements in exploratory behaviors but also via

increments, one can place an object in the center of the open-field. The number of entries into and the time spent in the center increases from exposure one to exposure two without affecting other indices of spatial habituation (Dai et al. 1995).

CRITICAL ASSESSMENT OF THE TEST

The open-field paradigm is a well validated, simple and time economical test, which has been widely used to examine the neurobiological foundations subserving spatial memory, general activity and emotionality in rodents with different approaches including: lesions, drugs, electrophysiology, neuroanatomy, and neurogenetics.

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F.3.3.2**Spatial Discrimination****PURPOSE AND RATIONALE**

In the simplest case of discrimination learning the animal distinguishes between two symmetric stimulus-response sets, the equal probability of which has been changes by differential reinforcement events. Position of the cues with respect to the animal's body defines the CS⁺ and CS⁻. Usually left-right discrimination is employed, while axial orientation of the body is ensured by the construction of the apparatus (Siegel 1969).

PROCEDURE

Rats and mice of both sex are used and maintained under standard conditions. The apparatus used is usually a simple T- or Y-maze, with an electrifiable grid floor. The last 10 cm of each arm are separated from the rest of the apparatus by a swing-door which prevent the animal from seeing the food cup or the plastic sheet covering the grid in the goal area. A fixed resistance shock source is connected to an automatically operated switch. In an aversively motivated spatial discrimination learning the animal is trained to escape and/or to avoid foot shocks by always going to the right. Training starts by allowing the animal to explore the apparatus. Then the animal is placed on the start and after 5 s electric shocks (0.5 s, 50 Hz, 1.0 mA) are applied at 3 s intervals. The animals are trained to a criterion. On the following day the animal is retrained to the same criterion. After a 60 min interval the safe goal area is shifted to the other arm of the maze and the discrimination is reversed.

EVALUATION

Errors are scored. An error means that the animal enters the wrong arm with all four legs. During retention the number of trials until the animal makes correct choices are counted.

CRITICAL ASSESSMENT OF THE METHOD

The ecology of rodents makes these animals specially proficient in spatial discrimination learning, which is usually mastered in a few trials. Most of the initial errors are not due to the inability of the animal to remember the correct solution, but rather to its tendency to explore alternative pathways.

MODIFICATIONS OF THE TEST

Barnes (1979) and others introduced the radial maze as a modification of spatial discrimination. This method is now well established and widely used.

Ingram et al. (1994a, b) recommended the Stone 14-unit T-maze to find new pharmacological approaches for cognitive enhancement.

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F.3.3.3**Spatial Learning in the Radial Arm Maze****PURPOSE AND RATIONALE**

Olton and co-workers have developed a spatial discrimination task for rodents that has been extensively used in learning and memory studies, and that has served as the basic task for one of the most important theories on the role of the hippocampus (Olton et al. 1979; Olton and Samuelson 1976). The rat uses spatial information provided by the distal cues in the room to efficiently locate the baited arms. The radial arm-maze allows the study of spatial reference and working memory processes in the rat. In reference memory procedures, information is useful for many sessions/days and may usually be needed during the entire experiment. On the contrary, working memory procedures have a major temporal component as the information presented in the maze (arms baited) is useful for one session but not for subsequent ones; the rat has to remember the information during a delay interval (min to h). Correct choices in the radial arm-maze are rewarded by food.

PROCEDURE

The apparatus is a wooden elevated eight-arm radial maze with the arms extending from a central platform 26 cm in diameter. Each arm is 56 cm long and 5 cm wide with 2 cm high rails along the length of the arm. The maze is well illuminated and numerous cues are

present. Food pellets (reward) are placed at the end of the arms. During the test, rats are fed once a day and their body weights maintained at 85% of their free-feeding weight to motivate the rat to run the maze. Animals are trained on a daily basis in the maze to collect the food pellets. The session is terminated after 8 choices and the rat has to obtain the maximum number of rewards with a minimum number of errors.

EVALUATION

The number of errors (entries to non-baited arms) are counted during the session.

MODIFICATIONS OF THE TEST

Depending on the hypothesis being tested in the radial arm-maze (1) animals can be trained extensively and then receive specific brain lesions (hippocampus, septum or fimbria-fornix); after recovery from the surgery the rats are re-trained to determine the cognitive ability or the speed of recovery as these lesions severely disrupt processing of spatial information (Olton et al. 1978). (2) In working memory studies animals are forced to obtain reward in specific arms (4 arms), and after a time delay they have to either return to the same arms (win-stay) or to avoid these arms and obtain the food in the rest of the arms (win-shift). (3) Animals are trained to find food in only one arm, and after a delay they are required to return to the same arm. Many versions of the maze exist with differences in the shape, arm-length or the number of arms (12 or 24 arm-mazes), but all of them are appetitively-motivated. An aversively-motivated maze was developed by locating an 8-arm maze in a water tank (Buresova et al. 1985). Extensive modifications on the arm-maze were also developed by Kesner and co-workers and are the basis of the multidimensional attribute theory that characterize mnemonic functions (Kesner 1980, 1986).

CRITICAL ASSESSMENT OF THE TEST

The radial arm-maze has been widely used to determine the neurobiological mechanisms underlying spatial learning in rodents and to evaluate the effect of drugs. The deleterious effects of scopolamine, atropine, ethanol, benzodiazepines, haloperidol, ketamine, PCP, as well as the facilitatory effects of physostigmine, nicotine, pramiracetam, picrotoxin, naloxone have been reported in the literature (Levin 1988). One of the disadvantages of the test is that hypothalamic lesions or the anorectic effect of certain drugs (amphetamine) affect the appetitive nature of the maze and animals do not master the maze for this reason.

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F.3.3.4

Visual Discrimination

PURPOSE AND RATIONALE

Vision is better than any other sensory system for the analysis of spatial relationships in the environment of the animal. From the retina to the cerebral cortex, the organization of the visual system ensures processing of visual information according to simple principles, i. e. by fitting the distribution of light over the receptive surface to elementary geometrical concepts and by comparing these patterns with images stored in the memory. Visual pattern recognition is one of the most challenging problems of contemporary neurophysiology and experimental psychology, with significant implications for mathematical and technical modeling of perceptual phenomena (artificial intelligence). For review and details see Grüsser and Klinke (1971) and Sutherland (1969). Experimental studies of pattern discrimination must take into account the visual capability of the given species and present the discriminanda under conditions compatible with light sensitivity and acuity of the eye. The constructions of the apparatus should ensure that the discriminanda are viewed from one optimum distance and for a sufficient period of time. A thorough discussion of the pattern discrimination technique is to be found in Munn (1950).

PROCEDURE

Rats and mice of both sexes are used and maintained under standard conditions. The apparatus consists of a square 10×10 cm start area separated by a Plexi-

glas sliding door from the choice area, which is connected by swing doors to the goal compartment. The grid floor in the starting and the choice areas is electrifiable. The stimulus (mostly plastic cards 8 × 8 cm) can be attached to the swing doors. The patterns are black on a white background and have different forms. The apparatus is illuminated by a dim light. The animal is placed into the apparatus with all doors open and allowed to explore it. Then it is placed in the start and after 5 s released by raising the Plexiglas door. After another 5 s, electric shocks (1 mA, 50 Hz, 0.5 s, 1/3 s) are applied until the animal escapes through either of the open doors to the safe goal compartment where it is left for some seconds. As soon as this preliminary step is mastered, the stimulus cards are inserted, the negative door is locked and the grid section in front of this door is electrified. The animal is trained to a criterion. On the next day the animal is retrained to the same criterion and retention is expressed in savings. Another parameter which can be used to evaluate the savings is the cumulative number of errors until the criterion is reached (Thompson 1969).

EVALUATION

The number of correct answers as well as the number of trials until the criterion is reached are counted.

CRITICAL ASSESSMENT OF THE METHOD

The method is time consuming and only useful to address a specific hypothesis.

MODIFICATIONS OF THE TEST

Steckler et al. (2000) and Van Gaalen et al. (2003) tested mice for their ability to learn and perform a series of simultaneous visual discriminations, which allowed a dissociation between accuracy of discrimination from those of motivation and behavioral disinhibition. Five operant chambers (20 × 15 cm) with curved rear walls (developed with TSE, Bad Homburg, Germany) were used. Set in the curved wall of each box was an array of five circular holes, 2 cm in diameter, 3 cm deep and 1.5 cm above floor level. Each hole was equipped with an infrared photocell beam crossing the entrance vertically, a yellow LED at the rear end of the hole, providing 5 lux illumination at the hole's entrance, and a food pellet dispenser which allowed delivery of a 20-mg dustless food pellet directly into the hole where a correct response has been made. Presentation of the light stimulus, the operandum and the food reward all in the same location was chosen to enhance stimulus–response–reward contiguity. Each hole could be closed separately by a metal sheet to pre-

vent access for the animal, if required. In addition, the chamber could be illuminated by a white house light (30 lux) mounted in the center of the roof. The boxes were housed in dark, sound-attenuating compartments. On-line control of the apparatus and data collection were performed using a special computer system. After habituation and autoshaping, the program was switched to five-choice discrimination and then to two-choice discrimination with increasing and decreasing response requirement.

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F.3.3.5

Spatial Learning in the Water Maze

PURPOSE AND RATIONALE

A task was developed where rats learn to swim in a water tank to find an escape platform hidden under the water (Morris 1984). As there are no proximal cues to mark the position of the platform, the ability to locate it efficiently will depend on the use of a configuration of the cues outside the tank. Learning is reflected on the shorter latencies to escape and the decrease on the length of the path to find the platform. Although rodents can find the platform by using non-spatial strategies, the use of a spatial strategy is the most efficient way to escape and young animals develop the spatial strategy after a small number of trials.

PROCEDURE

Different strains of rats are generally used (Long Evans, Wistar, Sprague-Dawley). The apparatus is a circular water tank filled to a depth of 20 cm with 25°C water (Brioni et al. 1990; Morris 1984). Four points equally distributed along the perimeter of the

tank serve as starting locations. The tank is divided in four equal quadrants and a small platform (19 cm height) is located in the centre of one of the quadrants. The platform remains in the same position during the training days (reference memory procedure). The rat is released into the water and allowed 60–90 s. to find the platform. Animals usually receive 2–4 trials per day for 4–5 days until they escape onto the platform. Well-trained rats escape in less than 10 s.

EVALUATION

The latency to reach the escape platform is measured during the training days. A free-swim trial is generally performed after the training days where the escape platform is removed and the animal is allowed to swim for 30 s. With the help of a video system, the latency to reach the previous position of the platform, the number of annulus crossings as well as the time the rat spent in the training quadrant are measured. Well-trained rats show short latencies, a large number of annulus crossings and bias to the quadrant where the escape platform was located during the training sessions.

MODIFICATIONS OF THE TEST

In the “cue” version of the water maze, the platform is clearly visible over the water and allows the evaluation of the motor and motivational aspects of the rat under study, as the animals should easily find the visible platform in 10–15 s. As circling or other non-spatial strategies can be used to find the platform, alternative measures of learning were developed (Gallagher et al. 1993), like the use of brief probe trials during training (rather than at the end), the use of proximity measures (an index of search error or deviations from the optimal path), and the learning index (proximity measures during weighted probe trials). Buresova and co-workers have developed the use of a collapsible platform to improve the accuracy of the water maze (Buresova et al. 1985). To evaluate working memory processes in the water maze, the rat can be trained to find a new platform position and later its performance is tested after a short delay (2–4 h). A spatial discrimination version of the water maze was also developed (Decker et al. 1992; Morris 1984), where the rat has to discriminate between two similar platforms based on spatial differences (one platform provides a mean to escape while the other sinks upon contact).

CRITICAL ASSESSMENT OF THE TEST

This test allows the researcher to study working and reference memory processes, and to dissociate the memory deficits induced by brain lesions or drug injec-

tions from the motor, motivational or sensory deficits. This test has been used to study the effect of drugs on memory (Brioni et al. 1991; Brioni et al. 1990. Decker et al. 1992), the participation of the hippocampus, septum, amygdala and nucleus basalis magnocellularis (Morris et al. 1986; Morris 1989), catecholamine depletions, as well as recovery of function (McNamara and Skelton 1993). It has been particularly sensitive to demonstrate that aged rats exhibit deficits in the acquisition of spatial information when compared to young or middle age controls (Rapp et al. 1987). The major advantages of the water maze over the radial arm-maze are: (1) that animals can be trained in a significantly shorter period of time (one week) while the arm-maze studies require several weeks of training; (2) intramaze cues like odor trails are eliminated in the pool; (3) large dose-response studies can be conducted in a week's time; (4) the motor or motivational problems in the rat can be detected in a cue trial; and (5) that the animals are not food-deprived during the test. From a theoretical point of view, the water maze is an aversively-motivated task while the arm-maze is appetitively-motivated.

MODIFICATIONS OF THE METHOD

Nitta et al. (1994, 1996; Nabeshima 1995) found an impairment of the performance of the water maze task in rats treated by infusion into the cerebral ventricle for 14 days with β -amyloid protein which consisted of senile plaques of Alzheimer's disease. The authors recommended that β -amyloid protein-treated rats as an animal model for Alzheimer's disease.

Song et al. (1997) reported that thymectomized rats, 5 weeks after surgery, showed a significant impairment of learning and memory as shown in deficits in passive avoidance and in the Morris water maze test.

Sun and Alkon (2004) found that induced depressive behavior impairs learning and memory in rats using an open space swim test. The model indexes searching activity of the animals, with the induced depressive immobility behavior showing specific sensitivity to three major prototypic classes of antidepressants and a selective serotonin reuptake inhibitor.

Zoladz et al. (2006) reported enhancement of long-term spatial memory in adult rats by the non-competitive NMDA receptor antagonists memantine and neramexane.

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- Stuchlik et al. (2004), and Stuchlik and Vales (2005) designed a pharmacological mode, which they called the active allothetic place avoidance (AAPA) task, a cognitive task which requires rats to separate two conflicting spatial reference frames (room and arena frames), when only the room frame is relevant for solution of the task. Stuchlik and Vales (2005) showed that systemic administration of MK-801 elicits a behavioral deficit in rats in the AAPA task irrespective of their intact spatial pretraining.

PROCEDURE

Male Long-Evans strain rats weighing 350–450 g were housed under constant temperature at a 12:12 light:dark cycle. Under light ether anesthesia, animals were implanted with a low-impedance connector made from a hypodermic needle, which pierced the rat's skin between the shoulders. The sharp end of the needle was then cut off and bent with tweezers to form a small loop, which prevented the connector from slipping out and provided anchor for an alligator clip, which was connected to a shock-delivering wire.

MK-801 was injected i.p. in doses of 0.15 or 0.2 mg/kg 30 min before each training session.

The AAPA task apparatus consists of a smooth metallic circular arena (80 cm in diameter) enclosed within a 30-cm-high transparent Plexiglas wall and elevated 1 cm above the floor. There were two arenas located in two different rooms. The upstairs room (4 m × 5 m) contained various extramaze landmarks. The downstairs room (5 m × 5 m) contained different visual cues organized in a different layout. At the beginning of each training session, a rat was placed on the rotating arena (1 rpm), where a directly imperceptible 60° to-be-avoided sector (shock sector) was defined by the computer-based tracking system, located in an adjacent room. The location of the shock sector could be determined exclusively by its spatial relation to distal orienting cues in the room. The rat wore an infrared light-emitting diode fixed between the shoulders with a light latex harness, and its position was tracked every 40 ms and recorded onto a computer track file, allowing subsequent reconstruction of the track with an off-line analysis program.

Whenever a rat entered the shock sector for more than 0.5 s, mild electric shocks (50 Hz, 0.5 s) were delivered in intervals of 1.5 s until the rat left the shock sector for at least 0.5 s. The shocks were delivered through a thin low-impedance wire implant on the back of the rat standing on the grounded floor. The appropriate shock current (ranging between 0.2 and 0.7 mA) was individualized for each rat to elicit a rapid

F.3.3.6

Active Allothetic Place Avoidance Task

PURPOSE AND RATIONALE

Bures et al. (1997), Fenton et al. (1998), Cimadevilla et al. (2000, 2001a, 2001b), Stuchlik and Bures (2002),

escape reaction but to prevent freezing. Since the arena was rotating, the rat had to move actively away from the shock in the direction opposite to the arena rotation, otherwise it was passively transported to the shock sector. Experimental sessions in AAPA lasted 20 min and each rat had one session every day, carried out during daylight hours.

Initially, non-injected rats were trained for four consecutive daily sessions to avoid a sector located in the north of the upstairs room to learn the task without any drug administration. Subsequently, in session 5 they were injected with either saline or MK-801 and tested for the effect of the drug on the well-pretrained place avoidance (reinforced retention). After a week-end break rats were trained daily under saline or MK-801 in the downstairs arena in order to test reacquisition of AAPA in a new environment, when they were already familiar with the procedural component of the AAPA task (sessions 6–10). The shock sector was defined in the south of the room in the downstairs arena.

Rats were divided in three experimental groups: controls, MK-801 0.15 or 0.2 mg/kg i.p., respectively.

EVALUATION

The following parameters were recorded and analyzed in order to assess rats' behavior in the AAPA: the total distance traveled in a session (DISTANCE) measured in the arena frame (which only takes into account active locomotion) reflected the locomotor activity of the rats, and the number of entrances in the shock sector (ERRORS) reflected the efficiency of spatial performance in the AAPA task. The maximum time a rat spent in the safe part of the arena between two ERRORS in a particular session was also recorded. It reflected the ability to remember the shock sector location and to avoid it. Data from the initial 4 days of intact pretraining in the upstairs room were analyzed using a two-way ANOVA (groups \times sessions) with repeated measures on sessions, data from the fifth day (retention session) were compared in a between-group fashion using one-way ANOVA, and data from the reacquisition training in the downstairs room were analyzed with a two-way ANOVA (groups \times session) with repeated measures on sessions. Tukey's HSD test was used when appropriate.

CRITICAL ASSESSMENT OF THE METHOD

The AAPA task has been used for various pharmacological studies. In this paper, the authors claim that testing the effect of various substances using the

AAPA task may be a promising way to predict their influence on cognitive symptoms.

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F.3.3.7

Olfactory Learning

PURPOSE AND RATIONALE

Odors provide rodents with important information on the environment and the learning of successive olfactory discrimination problems in rats is closely related to the acquisition rules of higher primates. Odor-reward associations are learned in few trials as odors exert more discriminative control over other sensory modalities like tones or lights (Nigrosh et al. 1957). Animals have to learn to discriminate an arbitrary designated positive odor (i. e., banana) from a negative one (i. e., orange) to receive a reward.

PROCEDURE

Rats (Sprague-Dawley or Long Evans) are generally used. In procedures described in the literature (Eichenbaum et al. 1992; Roman et al. 1993), animals are deprived of water for 48 h before the training and during the test they receive ad libitum water for only 30 min. The olfactory apparatus is a rectangular box (30 \times 30 \times 55 cm) with a photosensitive cell mounted

on top of the water spout/odor outlet. Rats are trained to approach the water spout and to brake the light beam. Responses to the positive odor are rewarded with water while responses to the negative odor results in the presentation of a light flash. The intertrial interval before the presentation of a new odor is usually 15 s. and the sessions last 30 min. per day. Sessions are terminated when the rat makes 90% correct choices or after 400 trials.

EVALUATION

The animal is rewarded with 0.05 ml of water when it brakes the beam to the positive odor or when it does not respond to the negative odor. Incorrect responses (“no go” to the positive odor or “go” to the negative odor) are followed by a flash and a longer intertrial interval). Results are reported as the % correct responses or as a logit transformation of the % correct/incorrect response ratio.

CRITICAL ASSESSMENT OF THE TEST

The anatomical connections from the olfactory bulbs to cortical as well as subcortical areas are fairly well known, and brain lesions that impair olfactory discrimination learning (Otto et al. 1991; Roman et al. 1993; Stäubli et al. 1984) could be used as models of amnesia. Systemic injections of scopolamine disrupts the performance of rats during long delays with no effect on immediate recall, data that are consistent with the effect of scopolamine in humans (Ravel et al. 1992). Antagonists of the NMDA receptor impair acquisition of odor discriminations and block LTP, an electrophysiological correlate of memory (Lynch and Stäubli 1991). The test can also be used to evaluate the cognitive effects of drugs such as ACTH analogs that facilitate the storage of olfactory information (Roman et al. 1989).

MODIFICATIONS OF THE TEST

A T-maze with controlled access to the reward compartment was developed (Ravel et al. 1992), that allows the test to evaluate working memory processes after time delays (1–3 min.).

Willer et al. (1992) examined the effects of a competitive NMDA agonist on the ability of rats to acquire potentiated aversions to the odor element of a taste-odor compound.

Larson et al. (1995) studied in rats the facilitation of olfactory learning by a modulator of AMPA receptors.

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F.3.3.8

Aversive Discrimination in Chickens

PURPOSE AND RATIONALE

One to 2-days old chicks have been extensively used to study learning and memory. The chicks are trained to discriminate between an aversive and a non-aversive stimulus (colored glass beads) in a single 10–15 s learning trial. Following this learning trial, retention is monitored over a comprehensive range of learning-retention intervals to chart the course and to differentiate possible stages of memory formation. Three behaviorally sequentially dependent stages have been identified: a short-term memory stage, lasting approximately 10 min after learning; an intermediate-memory stage, available between 20 and 50 min post-learning, and a long-term memory stage available from 60 min after learning. The three stages are behaviorally separated by transient retention deficits occurring at around 15 and 55 min post-training. Gibbs and Barnett (1976), Gibbs and Ng (1976, 1979), Rickard et al. (1994, 2001), Bennett et al. (1996), Ng et al. (1997) used a three-stage model to test the influence of drugs on memory formation.

PROCEDURE

Day-old, male, White-leghorn black-Australop chickens are housed at an ambient temperature of 28°C. In

a single passive avoidance learning trial, chicks are pre-trained to peck at a 4 mm chromed bead, dipped in water and presented for 10 s. A similar bead, dipped in the chemical aversant methyl anthranilate, is presented for 10 s. Data from chicks failing to peck the bead on this trial or not showing characteristic disgust responses after pecking are eliminated from subsequent data analysis. Retention is tested by presenting for 10 s a dry bead similar to that used for the pre-training trial.

In a discrimination paradigm, chicks are trained to avoid a red bead and are tested on red and blue beads successively and discrimination ratios registered.

In an additional discrimination paradigm, chicks are given two aversive beads – a chrome and a red bead. They are trained on one of the aversive beads sometime before drug treatment. The other aversive bead is associated with drug treatment and the chicks are subsequently tested with all three beads.

Twenty different chicks are used for each data point. Retention in both paradigms is indexed by the proportion of chicks avoiding the aversive bead, with the proviso that in the discrimination paradigm is indicated by the avoidance of the red bead and pecking on the blue bead. Retention is tested at various times between 5 and 180 min after learning or at 24 h.

Drugs are administered in 0.1 ml volumes subcutaneously into a fold of skin on the ventral side of the rib cage.

EVALUATION

Discrimination ratios of treated animals after various time intervals following administration of drugs are statistically compared with saline treated controls.

CRITICAL ASSESSMENT OF THE METHOD

The test has been used with slight modifications successfully by many authors in several research groups (e. g., Hölscher and Rose 1992; Sandi and Rose 1994; Stephenson and Andrew 1994; Zhao et al. 1994; Deyo and Hittner 1995; Crowe and Shaw 1997; Venero and Sandi 1997; Bennett et al. 1998; Tiunova et al. 1998; Rose and Stewart 1999)

MODIFICATIONS OF THE TEST

Bourne et al. (1991) compared the taste and odor aversant methyl anthranilate with the odorless quinine as averants.

Clements and Bourne (1996) and Stamatakis et al. (1998) injected drugs, e. g., GABA agonists or α -2-noradrenergic agonists and antagonists, directly into the intermediate median hyperstriatum ventrale of day-

old chicks, prior to training on a chrome bead dipped in either methyl anthranilate or quinine.

Colombo et al. (1997) injected enkephalins into 2 regions of the 2-day-old chick brain: the intermediate medial hyperstriatum and the lobus parolfactorius.

Tiunova et al. (1996) trained chicks to discriminate between edible chick crumbs and arrays of colored bead glued to the floor of their cage.

Gilbert et al. (1989) reported a simple, one-trial, learning paradigm in young chicks. Chicks were separated from their brood mates and placed in a small isolation chamber. A T-maze connected the isolation chamber to the brood space, allowing the chick to escape isolation stress and rejoin the brood. When the chick successfully negotiated the corridor, the latency to perform this task was recorded. On a subsequent trial, any improvement in the speed of performance was recorded to reflect the chick's memory of the task.

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F.3.4

Conditioned Responses

F.3.4.1

Conditioned Nictitating Membrane Response in Rabbits

PURPOSE AND RATIONALE

The rabbit's classically conditioned eyeblink response has become a widely used model system for studying associative learning in mammals (Gormezano et al. 1987) and to find drugs potentially useful in the treatment of age-related memory disorders (Ghoneim et al. 1994; Woodruff-Pak et al. 1994a, b, 1997; Solomon et al. 1988, 1995a, b).

PROCEDURE

Animals

New Zealand white albino rabbits weighing 2.0 kg are housed individually under consistent light with free access to food and water.

Apparatus and General Procedure

A small loop of surgical nylon is sutured into the right nictitating membrane, and the surrounding hair is re-

moved. One day later, the rabbit is placed in a Plexiglas restrainer, and two stainless-steel wound clips are applied to the skin over the parietal region. The rabbit is fit with a headmount that supports a photoresistive assembly for recording the nictitating membrane response by physical coupling with a length of thread to the nylon loop in the nictitating membrane. The transducer assembly converts nictitating membrane movements into electrical signals that are subjected to an analog-to-digital conversion using a 5-ms sampling rate and a resolution of 0.06 mm actual membrane extension. The animal is then positioned in a ventilated, sound-attenuated chamber facing a stimulus panel containing an 11.4-cm speaker and two 6-W, 24-V DC house lights, one mounted at each side of the speaker. During the course of the experiment, two stimuli are employed as conditioned stimulus: a) a 1000-ms, 1-kHz, 84-dB tone; b) a 1000-ms, intermittently presented light produced by interruption of the house lights at 10 Hz to yield a change in illumination, measured at the eye level of the rabbit from 32.11 to 8.01. The unconditioned stimulus is a 100-ms, 3-mA, 60Hz shock delivered to the wound clips by a constant current shock generator.

Drugs

Drug solutions or saline are injected subcutaneously into the cervical area of the rabbit via an infusion pump at a rate of 3 ml/min, 30 min before behavioral testing.

Procedure

Experimentally naive rabbits are randomly assigned in equal numbers to each of the treatments ($n = 10$ per treatment). The experiment consists of two phases: Phase 1 is an adaptation day followed by 9 days of acquisition training. No stimuli are presented during the 60-min adaptation session. Subjects are injected with their assigned treatment 30 min before each acquisition session. Each acquisition session consists of 30 tone-shock and 30 light-shock trials presented in a randomized sequence within 10 trial blocks, with the restriction that no more than three consecutive tone or light trials can occur. On each conditioned stimulus – unconditioned stimulus trial, the offset of the 1000-ms tone or light conditioned stimulus occurs simultaneously with the onset of the 100-ms unconditioned stimulus. The inter-trial interval is about 60 s. A response is defined as at least a 0.5-mm extension of the nictitating membrane. Responses occurring during the tone or light conditioned stimulus, but before the unconditioned stimulus are recorded as conditioned response;

those occurring after the unconditioned stimulus onset are recorded as unconditioned response.

EVALUATION

The data are analyzed by repeated measures analyses of variance and Tukey tests. The significance level is set at $p < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

The rabbit nictitating membrane response has been used widely to study the effects of drugs on learning (Schindler and Harvey 1990).

MODIFICATIONS OF THE TEST

Several authors used a corneal air puff as unconditioned stimulus (Robinson et al. 1993; Solomon et al. 1995, 1996).

Scavio et al. (1992) studied post-training effects of several drugs, such as amphetamine, chlorpromazine, ketamine, and scopolamine, on the acquisition and extinction of the rabbit's conditioned membrane response.

The effects of modulating tone frequency, intensity, and duration on the classically conditioned rabbit nictitating membrane response were studied by Kehoe et al. (1995).

Solomon et al. (1996) described behavioral paradigms for delay, trace, and long-delay conditioning.

Various drugs influence the nictitating membrane response in rabbits, such as morphine (McEchron and Gormezano 1991), cocaine (Marshall-Goodell and Gormezano 1991), sodium pentobarbital (Chen et al. 1992), haloperidol (Sears and Steinmetz 1990), MDA (3,4-methylenedioxyamphetamine) (Kirkpatrick-Steger et al. 1991; Romano and Harvey 1993), harmaline (Harvey and Romano 1993; Du and Harvey 1997), ketamine (Ghoneim et al. (1994), isoflurane (El-Zahaby et al. 1994), L-NAME (nitric oxide synthesis inhibitor, Du and Harvey 1996), serotonin antagonists and agonists (Welsh et al. 1998a, b), calcium antagonists (Woodruff-Pak et al. 1997), nitrous oxide (Ghoneim et al. 1999).

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F.3.4.2

Automated Learning and Memory Model in Mice

PURPOSE AND RATIONALE

Vanover and Barrett (1998) developed and validated pharmacologically an automated, relatively rapid, and reproducible behavioral model of learning and memory using an autoshaped procedure in mice. Autoshaping procedures were first described for pigeons by Brown and Jenkins (1968) and have been used subsequently as a means to assess learning and memory in monkeys (Sidman and Fletcher (1968), in rats (Steckler et al. 1993; Oscos et al. 1998), and in mice (O'Connell 1980).

PROCEDURE

Naive male mice weighing 25–35 g are randomly assigned to experimental groups. They are housed at approximately 22°C at a 12:12 light/dark cycle with food and water continuously available. Twenty-four hours before each test, mice are isolated into individual cages and all food is removed. Water remains continuously available. Tests are conducted on two consecutive days. Mice are fed 1.5 g food immediately after the first session (day 1).

For experimental sessions, mice are placed in sound-attenuating enclosures containing specially designed operant chambers (12.5 × 11 × 12.5 cm) equipped with a recess (dipper well; 2.2 cm diameter, 1.3 cm deep) for dipper accessibility on one wall of the chamber and two additional smaller holes (1.3 cm diameter, 0.9 cm deep) on either side of the dipper well. The dipper can be raised into the dipper well

for a sucrose solution (110 g/1.01 water) reinforcer presentation. Each recess has a photocell monitoring nose-poke responses. In addition, a speaker capable of sounding a stimulus tone is located on the wall opposite to the dipper and a house-light is on the ceiling. A computer and associated interface controls stimulus events and records nose-poke responses.

Five to 9 mice per group are tested in a 2-day procedure designed to measure acquisition and retention of nose-poke response under an autoshaping schedule of reinforcement. Experimental sessions are conducted at the same time each day for 2 consecutive days. Both the acquisition session (day 1) and the retention session (day 2) are identical.

During the autoshaping procedure which is used to measure acquisition and retention of the response reinforcement for nose-poking a tone is added. This tone sounds on a variable-interval schedule of presentation (mean of 45 s, range 4–132) and stays for either 6 s or until a nose-poke in the dipper well is made before the end of the 6 s period, at which time the tone is turned off and a dipper with sucrose solution is presented. If a mouse fails to make a dipper well nose-poke during the tone, the dipper is automatically presented at the termination of the tone. A dipper well nose-poke response made during the presence of the tone is counted as reinforced response. Dipper well nose-poke responses made while the tone is off are counted but have no consequence. Each session lasts for 2 h or until 20 reinforcers have been earned, whichever comes first. In addition to dipper well nose-poke responses, nose-pokes in the smaller holes to the left and right of the dipper are counted but have no consequence.

Drugs in various doses or vehicle are administered i.p. immediately before the session.

Rates of nose-poke responses made in the small holes on either side of the dipper well serve as a measure of general activity. The computer presents tones on a variable interval with a mean of 45 s and 10 possible intervals (4, 10, 15, 22, 29, 38, 49, 64, 87, 132 s) chosen randomly without replacement until all intervals have been used.

The latency of the response to the 10th reinforcer is considered as a measure of acquisition and retention, because all mice have been exposed to all possible intervals (in random order) by the 10th tone. In order to eliminate the variance due to the variability of the first reinforcer, it is necessary to adjust any measure of acquisition and retention according to the response latency to the first reinforcer. The 10th response latency measure is thus adjusted by subtracting the response latency to the first reinforcer (L-10-1) in order to en-

sure all mice have been exposed to the reinforcement contingency.

An other measure of acquisition and retention consists of the rate of nose-poke responding in the dipper well during the total session.

EVALUATION

The mean and standard error of the mean are calculated for every group. Two-tailed Student *t*-tests are used to compare two independent groups on any one dependent variable. In addition, two-tailed paired *t*-tests are conducted to compare data from day 1 to data from day 2 for any one group. One-way analyses of variance (ANOVA) are conducted across groups for effects of dose on acquisition (day 1 performance). One-way analyses of covariance (ANCOVA) are conducted across groups for effects of dose for retention (day 2 of performance) with the performance on day 1 as the covariate. When an ANOVA or ANCOVA shows statistical significance, post-hoc Duncan's multiple range tests are conducted with every group compared with vehicle. *P* values less or equal to 0.05 are considered statistically significant.

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F.3.4.3

Learning Deficits after Postnatal Anoxia in Rats

PURPOSE AND RATIONALE

Oxygen deprivation causes irreversible damage to the adult mammalian brain, but during perinatal development the nervous systems appears to be more tolerant to hypoxic events. Several animal models have been developed to study in detail the pathophysiology of perinatal hypoxia and its behavioral consequences (Speiser et al. 1988; Dell'Anna et al. 1991; Speiser et

al. 1991; Raju 1992; Buwalda et al. 1995; Roohey et al. 1997; Lebedev et al. 2003; Zhuravin et al. 2004). Speiser et al. (1998) tested the effect of a monoamine oxidase B inhibitor on cholinergic functions and behavior in rats submitted to postnatal anoxia.

PROCEDURE

Wistar rats were kept at $24 \pm 2^\circ\text{C}$ and a 12:12 h light:dark cycle with free access to food and water. For breeding, a male 4- to 8-month-old was kept with a pair of females, 2- to 5-months-old, for 10 days. The male was withdrawn, and the females were kept together for an additional 10-day period, and then housed in individual cages where they had a litter of about 9-12 pups. Mother and five to seven male offspring were kept together until weaning (21 days), then separated and housed five to seven to a cage.

Only male pups were used for anoxia. On the second postnatal day, five to six pups were placed in a glass container equipped with an inlet and outlet for the displacement with 100% nitrogen at near atmospheric pressure. After a stay of 30 min, the pups were removed to room air and resuscitated by soft intermittent massage to the chest, then returned to their mothers.

To avoid handling which could affect behavior, the test drug was administered in drinking water to the nursing mothers starting on the first day of delivery. Controls received tap water. After weaning and to completion of the study, the young rats were given access to drug in drinking water.

For evaluation, motor behavior was recorded at ages of 14, 21, 28, and 35 days. Retention of one-trial step-through passive avoidance was determined at the age of 45 days. Water-maze tests were performed at the age of 50-60 days.

EVALUATION

Data were analyzed by either ANOVA or the unpaired or paired two-tailed Student's *t*-test.

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F.3.5

Studies in Monkeys

PURPOSE AND RATIONALE

Nonhuman primates have the closest taxonomic relationship to humans, sharing many morphologic and physiologic similarities in the central nervous system. These similarities increase the likelihood that studies in aged nonhuman primates will provide information about drugs that is relevant to humans. Nonhuman primates offer additional advantages for neurobehavioral animal models of aging in that many of the behavioral processes thought to be affected by aging (e. g. reaction time, attention, learning and memory) can be studied easily in nonhuman primates. Evidence is beginning to accumulate suggesting that certain neurological and behavioral deficits are observed in aged human and in nonhuman primates (Bartus 1979; Struble et al. 1982; Wisniewski et al. 1973).

PROCEDURE

The apparatus developed specifically for the series of studies used to develop the primate model was the Automated General Experimental Device (AGED), the rationale upon which its development was based and its application to geriatric research have been discussed in detail elsewhere (Bartus 1979; Bartus and Dean 1981; Dean et al. 1983). The AGED is a totally automated, computer-controlled testing system, whose prominent feature consists of a 3 × 3 matrix of stimulus response (SR) panels. Each SR panel is hinged-mounted directly in front of the reinforcement well so that when a panel is pushed, a red switch is magnetically activated and a reinforcement well is exposed. Both colored and patterned stimuli can be projected

onto the SR panels. A plastic partition with a stimulus window and armholes separates the monkey from the SR matrix. The stimulus observation window is equipped with a photocell and an infrared light source to detect when the monkey's head is oriented toward the stimuli.

EVALUATION

The monkey must remember the stimulus location to get a reinforcement. Number of correct answers will be counted as well as the time until the monkey answers correctly.

CRITICAL ASSESSMENT OF THE METHOD

This system provides an accurate and objective means of collecting data under a number of behavioral paradigms. Further, this system provides experimental control over a number of variables that might confound behavioral measures (especially in drugged or aged subjects), thus simplifying the interpretation of differences observed and increasing the likelihood that these interpretations are accurate.

MODIFICATIONS OF THE METHOD

Cai and Arnstein (1997) described dose-dependent effects of Dopamine D1 receptor agonists on spatial working memory in aged monkeys.

Gandy and Walker (2004) proposed non-human primates for modeling hemorrhagic and encephalitic complications of Alzheimer amyloid- β vaccination and for assessing the efficacy and safety of immunotherapeutics in Alzheimer's disease.

Taffe et al. (2004) reported the development of a visuo-spatial paired-associate learning task in rhesus monkeys for early detection of symptoms of Alzheimer's type dementia.

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F.3.6

Electrophysiological Methods

F.3.6.1

Long-Term Potentiation in Hippocampal Slices

PURPOSE AND RATIONALE

Long-term potentiation (LTP) in the hippocampus is perhaps the most dramatic example of activity-dependent synaptic plasticity that has yet been identified in the mammalian brain (Landfield and Deadwyler 1988; Bashir et al. 1994). A brief tetanus to any one of a number of monosynaptic excitatory pathways in the hippocampus can enhance the amplitude of evoked responses in the tetanized pathway for hours or days thereafter. The fact that it occurs in the hippocampus has done much to stimulate interest in LTP as a synaptic model of memory, since the importance of the hippocampus for memory processing has been evident ever since the discovery that its bilateral removal in man causes a profound impairment in the ability to lay down new memories (Scoville and Milner 1957; Milner 1972). The particular popularity of the slice preparation prepared from the rodent hippocampus rests on its lamellar and laminar organization (Teyler 1980).

PROCEDURE

Transverse slices, 400 μ m thick, are cut from the hippocampus of male albino guinea pigs weighing 250–300 g and prepared for electrophysiological recordings (Teyler 1980; Tanaka et al. 1989, 1990). Slices are incubated for 90–120 min in the recording chamber to allow equilibration with artificial cerebrospinal fluid. They are submerged, placed on a nylon mesh and perfused at a flow rate of 2–2.5 ml/min with oxygenated (95% O₂/5% CO₂) cerebrospinal fluid having the following composition (in mM): NaCl 124, KCl 3.3, CaCl₂ 2.5, KH₂PO₄

1.25, MgSO₄ 2, NaHCO₃ 25.7, glucose 10. The recording chamber is maintained at 33 \pm 2°C. The extracellular population spike is obtained using glass microelectrodes filled with 2 M NaCl, which have resistances of 2–5 M Ω . The electrodes are placed into the stratum pyramidale of CA1 or CA3. The signal is amplified and stored on magnetic discs for later analysis. The evoked responses are averaged and analyzed off-line using a personal computer. The magnitude of the population spike is evaluated by taking the voltage difference between the negative peak and the following positive peak. Either mossy fibers in the hilus fasciae or commissural/associational fibers in the stratum radiatum are activated via bipolar, sharpened silver wire electrodes insulated except for the tips. Constant current pulses (100 ms) are delivered with a frequency of 0.2 Hz, only during the test intervals. The stimulation intensity is adjusted to elicit the population spike of about 40% and 80% of its maximal amplitude in CA1 and CA3, respectively. After the baseline is recorded for 10–20 min, LTP is induced by repetitive stimulation of 100 pulses at 20 Hz for 5 s in CA1 and at 50 Hz for 2 s in CA3 at the same strength as for the test pulses. Responses by test pulses are recorded 0, 10, 20 and 30 min after repetitive stimulation. The test drugs are dissolved in the artificial cerebrospinal fluid and applied extracellularly at various concentrations by switching perfusion reservoirs.

EVALUATION

The time course of LTP is registered for CA1 and CA3. The mean percent increase in the amplitude of the population spike from baseline responses after drug application is compared with controls.

MODIFICATIONS OF THE METHOD

Fujii et al. (1997) studied the effects of an adenosine A₁ receptor antagonist, 8-cyclopentyltheophylline, on the reduction of long-term potentiation in CA1 neurons of **guinea pig** hippocampal slices. Reduction of long-term potentiation (depression) was achieved by delivering a train of low-frequency afferent stimuli 20 min after the tetanus.

Behnisch and Reymann (1993) employed slices of hippocampal area CA1 in the **rat** to test the hypothesis that the activation of metabotropic glutamate receptors during tetanization is necessary for the maintenance of long-term potentiation.

Akhondzadeh and Stone (1995) studied the phenomenon of hippocampal long-term depression. Extracellular recordings were made in the CA1 pyramidal cell layer of **rat** hippocampal slices following ortho-

dromic stimulation of Schaffer collateral fibres in stratum radiatum.

Oomura et al. (1996) measured long-term potentiation in hippocampal slice preparations after a brief tetanic stimulation at the Schaffer collateral/-commissural afferents of senescence-accelerated mice which were treated from 3 weeks of age up to 10 months either with subcutaneous injections of acidic fibroblast growth factor or saline.

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F.3.6.2

Long Term Potentiation in Vivo

PURPOSE AND RATIONALE

Placing recording and bipolar stimulating electrodes in the granule cell layer of the dentate gyrus and angular bundle allows the evaluation of long-term-potentiation *in vivo*, even in freely moving animals, and the comparison with effects on learning.

PROCEDURE

Female Sprague Dawley rats weighing 225–250 g are anesthetized with 1.5 g/kg urethane (i.p.) and placed in a Kopf stereotaxic instrument. The recording and bipolar stimulating electrodes are placed in the granule cell layer of the dentate gyrus and angular bundle, respectively (from bregma and cortical surface: Dentate: A.P. –4.0 mm, M.L. +2.4 mm, D.V. 3.0 mm. Angular bundle: A.P. –7.9 mm, M.L. +4.0 mm, D.V. –3.0 mm, incisor bar –5.0 mm). The recording electrodes are pulled from thin-walled glass capillary tubes, filled with 150 mM NaCl, and adjusted to resistances ranging from 2.0 to 4.0 MΩ. Stimulating electrodes are made from twisted nichrome wire with Teflon insulation, and approximately 0.75 mm separated each tip. The recording electrode is first lowered into area CA1 of the dorsal hippocampus. The stimulating electrode is then placed into the ipsilateral angular bundle. As the recording electrode is lowered further, field potentials are evoked to determine when the recording electrode enters the dentate granule cell layer.

All rats are maintained at 37°C. Responses are evoked using a Grass S-88 stimulator and a Microprobe System M-7070A amplifier, and are recorded on a Nicolet 310 oscilloscope.

Once the electrodes are appropriately placed, field potentials are generated over a range of stimulus intensities to generate an input/output (I/O) curve (pulse duration = 1 ms). The field potentials are quantified in two ways: The population spike (PS) is expressed as the distance from the deepest point (in mV) of negativity to the preceding highest positivity on the left and right side of the response, and then averaged. The slope of the rising phase of the population excitatory postsynaptic potential (pESP) is measured in mV/ms. The amplitude of the test pulse is based on the I/O

curve being 25% of the current intensity which evokes the maximal population spike. Long-term-potential is induced using three theta-burst stimulus trains, each delivered 1 min apart. Each train consists of five groups of four pulses at 100 Hz, separated by an interval of 150 ms. Each train is delivered at the stimulus intensity which evokes the maximal population spike at the I/O curve.

At the end of the experiment, the electrode placements are verified using standard histological techniques.

EVALUATION

Student's *t*-tests are used to assess the significance of differences between the means of both the population spike amplitudes and the pESP slopes.

CRITICAL ASSESSMENT OF THE METHOD

Hölscher et al. (1997a, 1997b, 2002) argued that high-frequency stimulation-induced long-term potentiation and low-frequency stimulation-induced depotentiation in area CA1 of the hippocampus are not good models for learning.

MODIFICATIONS OF THE METHOD

Errington et al. (1987) perfused a NMDA receptor antagonist through a push-pull cannula into the dentate gyrus of anesthetized **rats** in order to observe its effect on the induction and maintenance of long-term potentiation and on the increase in release of endogenous glutamate associated with long-term potentiation.

Bennett et al. (1992) recommended cytochrome oxidase inhibition as an animal model of Alzheimer's disease. Rats were infused chronically with the selective inhibitor of cytochrome oxidase, sodium azide, delivered via subcutaneously implanted minipumps. The azide treatment impaired both spatial and non-spatial learning. Further, the azide treatment inhibited a low-threshold form of hippocampal long-term potentiation, primed burst potentiation.

Croll et al. (1997) used this model to study time course and corresponding pathology of learning deficits in rats.

Brakebusch et al. (2002) found that mice deficient in brevican (a brain-specific proteoglycan) displayed impaired hippocampal CA1 long-term potentiation but showed no obvious deficits in learning and memory.

Namgung et al. (1995) described the characteristics of long-term potentiation in the intact **mouse**. Perforant path stimulation evoked both a population excitatory postsynaptic potential and a population spike

potential from the hippocampal dentate gyrus in urethane anesthetized animals. Long-term potentiation, as measured by increased population-spike amplitude and population excitatory postsynaptic potential slope, was successfully induced and reliably maintained at a stable level for at least 12 h.

Davis et al. (1997) described a simple method for inducing and monitoring long-term potentiation at perforant path-granule cell synapses in the dentate gyrus of freely moving mice using readily available miniaturized components. Tetanic stimulation induced long-term potentiation of the field excitatory postsynaptic potential and the population spike which persisted for more than 24 h but was not present 10 days after the tetanus.

Jibiki et al. (1993) and Kubota et al. (1994) studied the drug-induced blockade of induction of long-term potentiation in perforant path-dentate gyrus pathway in chronically prepared **rabbits**.

Gutnikov and Gaffan (1996) studied the effects of a NMDA receptor antagonist on memory acquisition and retrieval of visual-reward associations in the object-in-place memory task and on NMDA neurotoxicity in **rhesus monkeys**.

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F.3.6.3

Long Latency Averaged Potentials

PURPOSE AND RATIONALE

Wirtz-Brugger et al. (1986, 1987) studied long latency averaged evoked potentials (P300) in anesthetized rats as a possible model for detecting memory-enhancing drugs. The P300 waveform is described as a positive long-latency (~300 ms) potential believed to reflect endogenous cognitive processes rather than exogenous physical parameters of the stimulus. Compounds that have been shown clinically to enhance cognitive ability also significantly increase the integrated area under the P300 wave.

PROCEDURE

Male Wistar rats weighing 300–500 g are anesthetized with 120 mg/kg i.p. Inactin. Stainless steel screws serve as recording electrodes located at the surface of the cortex of seven distinct brain areas: posterior: Pz (midline), P3, P4 (lateral); central: Cz (midline); and frontal: Fz (midline), F3, F4 (lateral). A linked reference is provided with platinum needle electrodes behind the ears. All leads are fed into a digital averaging computer. Event-related potentials are elicited in response to an oddball paradigm of two tones (500 Hz frequent and 3 KHz rare) randomly presented with a probability of 10% for the rare tone. The intensity of the tones is 95 dB, pulse duration 100 ms, rise/fall 9.9 ms. The auditory stimuli are delivered bilaterally at a rate of 0.3/s through special Nicolet tubal tip in-

serts into the ear of each subject. One repetition of the paradigm consists of 300 tones. It is presented twice, before and after drug application. Evaluation of the rare responses consists of defining and comparing the P300 in terms of integrated area before and after drug. P300 area is calculated by integration of area under the curve.

EVALUATION

Values are expressed as means and standard error per group and percent changes from control. Statistical evaluation consists of paired *t*-tests to demonstrate significant differences.

MODIFICATIONS OF THE METHOD

Caudle (1993) demonstrated long latency potentials in the CA1 regions of the rat hippocampal slice suggesting that the *in vitro* P3 is an *in vitro* version of the long latency evoked potential known as the P300 in electroencephalogram studies.

Ikeda et al. (1995) studied the effect on a nootropic compound on event-related potential P300 in rats with lesions of the nucleus basalis magnocellularis.

Antal et al. (1994) studied the influence of cholinergic agents on visual discrimination and P300 in the behaving **monkey**. P300 latency was increased following scopolamine administration. Acetyl-L-carnitine increased P300 amplitude and decreased its latency.

In **squirrel monkeys**, clonidine significantly decreased the area and increased the latency of P300-like potential (Swick et al. 1993).

Kaga et al. (1992) studied P300 and choline acetyltransferase immunohistochemistry in septohippocampal neurons of **cats**.

Wang et al. (1999) reported that P300-like potential depends on muscarinic receptor activation in **rabbits**.

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F.3.7

Metabolic Influence

F.3.7.1

Sodium Nitrite Intoxication (NaNO₂)

PURPOSE AND RATIONALE

The manipulation of brain metabolism was used to show the beneficial effects of substances which influence learning and memory. Gibson et al. (1976, 1978), during investigations of sodium-nitrite (NaNO₂) on brain metabolism, demonstrated a close relationship between oxidative metabolism and cholinergic function. From the results of their studies, the possibility cannot be excluded that an induction of impairment of the cholinergic transmission in addition to a deficiency in brain metabolisms was induced (Schindler et al. 1984).

PROCEDURE

Male or female mice are used and maintained under standard conditions. Chemical hypoxia is induced by the injection of sodium nitrite (NaNO₂: 250 mg/kg s.c.), which reduces the oxygen-carrying capacity of the blood by converting hemoglobin to methemoglobin. This lethal dose (lethality 100% of controls) is injected 60 min after drug treatment. Immediately after the NaNO₂-injection the animals are placed in small Makrolon cages and the time between injection of NaNO₂ and cessation of respiration is recorded (Hock 1993).

EVALUATION

The time between injection of NaNO₂ and cessation of respiration is recorded. The prolongation of survival time is expressed in percent.

MODIFICATIONS OF THE METHOD

Using lower doses of NaNO₂ mice are submitted to a positive reinforcement paradigm (Schindler et al. 1984). Groups of 10 mice are water deprived for 24 h.

The mice are treated with the test compound 45 min before they are placed individually in a large chamber. On one wall of the chamber, there is a small compartment that contains a water bottle. The mouse easily finds the bottle and is allowed to drink for 30 s. Each mouse then receives a subcutaneous injection of 75 mg/kg NaNO₂ before being returned to the home cage. Twenty-four hours later, retention testing is performed by placing the mouse in the large chamber, but at this time, the small compartment is kept empty. The duration and frequency of the mouse's exploration of the small compartment while searching for water is evaluated over a period of 3 min. An increase in duration and frequency correlates with improved learning.

The tight rope test (Barclay et al. 1981) was used to test the effect of NaNO₂ with and without treatment on performance in mice (Peterson and Gibson 1982; Gibson et al. 1983).

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F.4

Animals with Memory Deficits

F.4.1

Memory Deficits After Cerebral Lesions

PURPOSE AND RATIONALE

Cerebral lesions have been used as a method of determining the involvement of a particular brain area in performing a particular function. By training an animal to perform a certain task, then lesioning a specific brain area, one can determine whether that area of the brain

is necessary or sufficient to perform that function; alternatively one can lesion before training and measure the rate of acquisition to determine the involvement of a brain area in learning a particular task.

Memory impairment has been produced by lesions caused by bilateral injections of ibotenic acid into the basal forebrain of rats. Water-maze tasks, habituation tasks, passive avoidance tasks with a light/dark compartment apparatus, and inhibition of the decrease of choline acetyltransferase activity in the cortex can be used to evaluate the effect of drugs (Fuji et al. 1993a).

PROCEDURE

Male Wistar rats weighing 270–310 g are anesthetized with sodium pentobarbital (45 mg/kg i.p.) and placed in a stereotaxic apparatus. Neurotoxic lesions of the basal forebrain are produced by injection of ibotenic acid. An injection needle connected to a 5- μ l microsyringe is inserted into the basal forebrain, identified according to the Paxinos and Watson (1986) atlas of rat brain (1.5 mm posterior, 2.8 mm bilateral to the bregma, 7.3 mm below the dura). Ibotenic acid is dissolved in 50 mM Na phosphate buffer at a concentration of 12 μ g/ml, and then 0.5 ml (6 μ g per side) is infused for 5 min. The injection needle is left in place for an additional 5 min to allow the toxin to diffuse away from the needle tip. One week later, the contralateral side is treated in the same manner. The same procedure is used to administer microinjections of 50 mM Na phosphate buffer into the basal forebrain of sham-operated rats. The lesion sites are mainly distributed in the ventromedial globus pallidus.

Then, 3–5 weeks after the first lesion, the animals are tested on the acquisition of a task in a Morris water maze (Morris 1981), on a habituation task in a novel situation, and on a passive avoidance task with light and dark compartments. The rats are treated once a day during the experiment.

After the behavioral experiments, the animals are sacrificed for determination of choline acetyltransferase activity in the brain according to Fonnum (1975). The tissue is homogenized (4% w/v) in cold 50 mM Na phosphate buffer (pH 7.4), and Triton X-100 (0.55, v/v) is added to homogenates to ensure enzyme release. To 75 μ l of enzyme solution, 125 μ l of substrate mixture {0.4 mM [14 C]acetyl-Co A (50.6 mCi/mmol or 1.87 GBq/mmol), 300 mM NaCl, 50 mM Na phosphate buffer (pH 7.4), 8 mM choline chloride, 20 mM EDTA-2Na, and 0.1 mM physostigmine} is added in a scintillation vial and the mixture is incubated at 37°C for 30 min. After the incubation, 0.8 ml of cold 50 mM phosphate buffer, 0.5 ml of ace-

tonitrile containing 2.5 mg of tetraphenylborate and 2.0 ml toluene are added to the scintillation vial. The vials are shaken lightly and allowed to stand overnight before radioactivity is determined.

EVALUATION

Data are evaluated by usual statistical means. All analyses are followed by a Bonferroni's test.

MODIFICATIONS OF THE METHOD

Although usually permanent, temporary lesions can be made by cooling the tissue of interest or by injecting a local anesthetic. Permanent lesions are of three primary types: **aspirative, electrical, and chemical** (McDonald and Overmier 1998).

Aspirative lesions are made by sucking out the tissue of interest. Lesions of this type are not selective with respect to tissue type. Neurons, support cells, and fibers coursing through the area are all eliminated.

Electrical lesions are also non-selective, i. e., they destroy all tissue types in the lesion area, including cell bodies, support cells, and fibers of passage. Harder et al. (1996) performed bilateral transection of the fornix in young adult **marmosets** producing a specific pattern of cognitive deficits, notably a lack of ability to recall visuospatial tasks learnt preoperatively, and a deficit in acquiring new visuospatial tasks following transection. This impairment could be ameliorated by cholinergic agonists and 5-HT_{1A} antagonists.

Ennaceur (1998) studied the effects of electrolytic lesions of the Medial Septum/Vertical Diagonal Band of Broca, the Globus Pallidus and the Substantia Innominata/Ventral Pallidum on the performance of **rats** in object-recognition memory and radial-maze learning tasks.

Chambers et al. (1996) proposed lesioning of the ventral hypothalamus in neonatal rats as an experimental model of schizophrenia with learning deficits.

Chen et al. (1997) observed a remarkable impairment of passive avoidance response by bilateral dorsal hippocampal lesions in rats.

In several studies **monkeys** were used:

Gaffan (1994) described a model of episodic memory impairment in monkeys with fornix transection.

Alvarez et al. (1994) studied memory functions in monkeys with lesions of the hippocampal formation and adjacent cortex.

Chemical lesions are induced by injecting chemical agents into specific brain sites. Chemical agents induce a more selective lesion, typically destroying cell bodies and dendrites while sparing axons (Coyle et al. 1981; Coyle 1983; Choi 1992).

The most commonly used neurotoxins in memory research are **excitatory amino acid neurotransmitters**, such as glutamate, and glutamate analogs such as ibotenate, NMDA, kainate, quisqualate and AMPA (Dunnet et al. 1989; Connor et al. 1991). These excitotoxins vary in their selectivity for the types of tissue damaged. For example, glutaminergic receptors in the hippocampal CA3 pyramidal cell layer are predominantly of the kainate subtype (Monaghan et al. 1983), and thus kainate administration induces more damage at this site than in areas of the hippocampus, in which kainate receptor density is not as great. In contrast, glutamate receptors in the dentate granule cells are primarily of the quisqualate and NMDA subtypes. As a result, they are relatively insensitive to damage by kainate administration, but highly vulnerable to ibotenate insult (Köhler and Schwarcz 1983).

Ishikawa et al. (1997) reported hippocampal-degeneration-inducing impairment of learning in rats after neonatal administration of monosodium glutamate or oral administration of trimethyltin.

Misztal et al. (1996) produced a short-term working memory deficit in rats by intraventricular infusion of quinolinic acid via ALZET osmotic minipumps for 2 weeks.

Zalewska-Wińska and Wśniewski (2000) tested the influence of (S)-3,5-DHPG, a selective agonist of group 1 metabotropic glutamate receptors, on the activity of the central nervous system in rats.

Zajaczkowski and Danysz (1997) induced deficits of spatial learning in rats by entorhinal cortex lesions with quinolinic acid.

Yamamoto et al. (2003) and Ahmed et al. (2004) infused ibotenic acid into the nucleus basalis magnocellularis of rats in order to establish a model of Alzheimer's disease.

Spowart-Manning and van der Staay (2005) found spatial discrimination deficits in the Morris water escape task in rats after bilateral lesions of the entorhinal cortex by injections of ibotenic acid.

Car and Wśniewski (1998) evaluated the effect of intracerebroventricular injection of baclofen and AP-7 on behavior in rats.

Two types of chemical lesions are used to examine the role of the cholinergic system in learning and memory. Ethylcholine mustard aziridinium ion, or **AF64A**, is a neurotoxin that is specific to cholinergic cells (Mantione et al. 1981). However, some non-cholinergic cells may also be destroyed. A neurotoxin more specific to cholinergic cells in the basal forebrain was described by Wiley et al. (1991). The compound, ^{192}IgG -saporin, is a monoclonal antibody to

the low-affinity nerve growth factor receptor (^{192}IgG) conjugated to saporin, a ribosome-inactivating protein. It specifically destroys cholinergic cell bodies in the nucleus basalis Meynert and the medial septal area. Cholinergic cells in the striatum, ventral pallidum, and thalamus are spared, as are non-cholinergic cells.

Fuji et al. (1993b) studied autoradiographically the influence of drugs on alterations in muscarinic cholinergic receptor [^3H]QNB binding induced by basal forebrain lesion in rats.

In order to confirm the antisense effects of sigma $_1$ (σ_1) receptor agonists on memory, Maurice et al. (2001) administered a phosphorothioate-modified antisense oligonucleotide and a mismatched analog intracerebroventricularly to mice.

Brandeis et al. (1995) induced deficits in cognitive functioning by stereotaxic application of AF64A (3 nmol/2 μl per side) in both lateral cerebral ventricles in rats.

Carli et al. (1997) caused impairment of spatial learning in rats by intrahippocampal administration of scopolamine or 7-chloro-kynurenic acid.

Abe et al. (1998) investigated the effect of a benzodiazepine receptor partial inverse agonist on the impairment of spatial memory in basal forebrain-lesioned rats.

Stancheva et al. (1993) studied in rats the effect of neonatal 6-hydroxydopamine treatment on learning and retention and on the level of biogenic monoamines in some brain structures as well as the influence of nootropic drugs.

Nitta et al. (1997) reported that the continuous infusion of anti-nerve growth factor monoclonal antibody into the septum of rats produces an impairment of memory.

Inagawa (1994) reported impairment of spatial working memory in rats induced by intracerebroventricular injection of the cholinergic neurotoxin, ethylcholine mustard aziridinium picrylsulfonate.

Bjugstad et al. (1998) gave chronic intraventricular infusions of tumor necrosis factor alpha to rats and found symptoms resembling the AIDS dementia complex in humans, including learning and memory impairment.

Beers et al. (1995) described spatial recognition memory deficits in Lewis rats following encephalitis after intranasal inoculation with herpes simplex virus type 1.

Harkany et al. (1998) showed that bilateral injection of β -amyloid [$\text{Phe}(\text{SO}_3\text{H})^{24}$]25–35 in rat nucleus basalis magnocellularis induces behavioral dysfunction.

tions, impairs learning and memory, and disrupts cortical cholinergic innervation.

Miguel-Hidalgo et al. (1998) induced specific degeneration of a large part of neurons in the lateral blade of the gyrus dentatus by small intrahippocampal injections of water with or without β -amyloid 1–28 fragment.

Ogasawara et al. (1999) studied the effects of NS-105, a cognition enhancer, on memory disruption by scopolamine, electrolytic lesion of the nucleus basalis magnocellularis, as well as by AF64A-, baclofen-, cerebral ischemia- and electroconvulsive shock-induced memory disruption in rats.

Baldi et al. (1999) studied the effects of combined medial septal area, fimbria-fornix and entorhinal cortex **tetrodotoxin** inactivations on passive avoidance response consolidation in the rat. Functional inactivation of these brain areas was induced by administration of 5 ng tetrodotoxin dissolved in 0.5 μ l saline according to the respective stereotaxic coordinates.

Intracerebroventricular injection of **streptozotocin**, in a subdiabetic dose to rats, causes prolonged impairment of brain glucose and energy metabolism. This is accompanied by impairment in learning and memory (Lannert and Hoyer 1998; Sharma and Gupta 2000, 2002).

Razani et al. (2001) reported that intracerebroventricular infusion of the 5-hydroxytryptamine_{1A} receptor agonist 8-hydroxy-2-(di-*N*-propylamino)tetraline (8-OH-DPAT) in rats induced a deficit in passive avoidance retention, which was attenuated by intraventricular galanin infusion.

Tchekalarova et al. (2003) showed that intracerebroventricular infusion of sarmesin, an angiotensin II analog, decreased seizure susceptibility, memory retention, and nociception in rats.

Rall et al. (2003) demonstrated that infusion of a **cyclooxygenase-2 inhibitor** into the dorsal hippocampus attenuates memory acquisition in rats.

Yu et al. (1997) reported significant impairment of learning and memory ability in **mice** after hippocampal microinfusion with colchicine.

Chapman et al. (1998) observed spatial working memory impairment in apolipoprotein E-deficient mice.

English et al. (1998) showed that infection with the LP-BM5 murine leukemia virus causes an AIDS-like syndrome – murine acquired immunodeficiency syndrome – in C57Bl/6 mice and impairs spatial learning without gross motor impairment.

Hiramatsu and Inoue (1999) studied the effects of nocistatin on nociceptin-induced impairment of learn-

ing and memory in mice. The drugs were injected into the lateral ventricle of the mouse brain in a volume of 5 μ l/mouse under brief ether anesthesia.

Fernandez-Ruiz et al. (1995) studied the long-term cognitive impairment in MPTP-treated **rhesus monkeys**.

Ye et al. (1999) evaluated a reversible acetylcholinesterase inhibitor for its ability to reverse the deficits in spatial memory produced by scopolamine in young adult monkeys and those that are naturally occurring in aged monkeys in a delayed-response task.

Yoneoka et al. (1999) established an adult rat model for the late onset of **radiation-induced cognitive dysfunction** in adult rats. Rats were irradiated with a total dose of 40 Gy, given as eight fractions in 24 days. At 12 months after irradiation, the passive avoidance task revealed a deterioration of the cognitive function.

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F.4.2

Cognitive Deficits After Cerebral Ischemia

PURPOSE AND RATIONALE

Cerebral vessel occlusion has been used as a model for stroke and multi-infarct cerebral dysfunction (see A.8.1).

Impairment of cerebral metabolism induced by reduced blood supply is known to induce cognitive deficits (Gibson et al. 1981). Because of the absence of posterior communicating arteries in the brain of **Mongolian gerbils**, complete forebrain ischemia can be produced by occluding both common carotid arteries resulting in amnesia (Levine and Sohn 1969; Schindler 1983; Schindler et al. 1984; Chandler et al. 1985; Lundy et al. 1986).

PROCEDURE

Male Mongolian gerbils (strain Hoe:Gerk) weighing 50–70 g are anesthetized by i.p. pentobarbital injection. Both common carotid arteries are exposed through a ventral neck incision and occluded for 5 or 10 min with miniature aneurysm clips. In sham-operated controls, the common carotid arteries are exposed but not occluded. Then, 24 h after occlusion, each animal is placed in the bright part of a light/dark-chambered apparatus for training. After a brief orientation period, the gerbil enters the second, dark chamber. Once inside the second chamber, the door is closed which prevents the animal from escaping, and a 100-V, 2-s foot shock is applied through the grid floor. The gerbil is then returned to the home cage. Testing is repeated 24 h later by placing the animal again in the bright chamber. The latency in entering the second dark chamber within a 5-min test session is measured electronically. The latency compared with sham-operated controls is decreased depending on the duration of ischemia. After drug treatment, an increase of latency before entering the dark compartment indicates good acquisition.

EVALUATION

Using various doses a dose-dependent increase of latency can be found after active drugs, sometimes resulting in inverse U-shaped dose–response curves.

Tamura et al. (2001) described the effect of permanent middle cerebral artery occlusion on cognitive impairments in **rats**.

PROCEDURE

Rats are anesthetized with 2% halothane in a gas mixture of 30% O₂ and 70% N₂O, then maintained with

1% halothane. Under anesthesia, the head is placed in a stereotaxic apparatus and the skin is incised between the external foramen and external canthus. A small hole is bored in the cranial bone under a surgical microscope. The dura is dissected and the middle cerebral artery exposed. The middle cerebral artery is electrically coagulated from its origin using a bipolar forceps. In sham-operated rats, the dura is exposed but the middle cerebral artery is not occluded.

MODIFICATIONS OF THE METHOD

Using a similar method, Yamaguchi et al. (1995) found that a muscarinic agonist improves the impairment of learning behavior in rats. Yonemori et al. (1996) tested spatial memory disturbances after focal cerebral ischemia in rats.

Noda et al. (1995) used a somewhat modified technique of permanent occlusion of the middle cerebral artery. Rats were anesthetized with halothane 2%. After the temporal muscle was retracted via a transretroorbital approach without removal of the temporalis muscle and zygomatic arch, the animals underwent a left subtemporal craniotomy. The stem of the middle cerebral artery was electrocauterized just medial to the olfactory tract and was cut to ensure the completeness of the vascular occlusion.

Another technique was described by Borlongan et al. (1995). Rats were anesthetized with 60 mg/kg pentobarbital, then shaved on the ventral portion of the neck. A V-shaped incision was made beginning at the caudal end of the sternomastoid and sternothyroid muscles, cutting towards the ears. After dissection, the right common carotid artery was exposed and carefully separated from the vagus nerve. The external carotid artery was then tied off tightly with two 5.0 silk sutures. The internal carotid artery was tied loosely and a small clamp was placed distal to the tie. Upon clamping off the common carotid artery, a small cut was made in the external carotid artery just above the bifurcation. The embolus was then introduced through the external carotid artery and guided into the internal carotid artery until resistance was felt. Mean length of the embolus inserted was 18–22 mm. The embolus, which was made by coating a 30-mm piece of 4.0 silk suture with silicone mixed with hardener, blocked the origin of the right middle cerebral artery. The embolus was left in place for 1 h. After removal of the embolus, the entry point of the embolus was cauterized, and the skin sutured.

Plaschke et al. (1999) used a model of permanent brain vessel occlusion originally described by Pulsinelli and Brierley (1979) to study the interrela-

tion between cerebral energy metabolism and behavior in rats.

Kimura et al. (2000) investigated the pathogenesis of vascular dementia in stroke-prone spontaneously hypertensive rats.

Hattori et al. (2000) tested cognitive deficits after focal cerebral ischemia in mice. Adult male C57/BL6 mice were anesthetized with 1%–1.5% halothane in oxygen-enriched air delivered by a face mask, then subjected to 60 or 90 min of intraluminal middle coronary artery occlusion or sham surgery. Unilateral middle coronary artery occlusion was achieved by introducing a 6-0 nylon monofilament into the right internal carotid artery through the external carotid artery, then positioning the filament tip for occlusion at a distance of 6 mm beyond the internal artery-ptyergopalatine artery bifurcation. Then, the wound was closed and the animal was allowed to emerge from anesthesia in its home cage. After 60 or 90 min of occlusion, the animals were briefly re-anesthetized with halothane, and the monofilament was removed. Cognitive deficits were evaluated by several behavioral tests and in the elevated plus-maze test.

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F.4.3

Strains with Hereditary Memory Deficits

Several animal strains have been recommended to study age-dependent memory deficits, such as the **senescence-accelerated mouse (SAM)** (Takeda et al. 1981, 1994, 1996, 1997; Fujibayashi et al. 1994; Maurice et al. 1996; Nishiyama et al. 1997; Flood and Morley 1998; Markowska et al. 1998). SAM includes accelerated senescence prone SAMP (SAMP1, P2, P3, P6; P7, P8, P9 and P10) and accelerated senescence-resistant mouse SAMR (SAMR1, R2 and R4) (Hosokawa et al. 1984; Yagi et al. 1998). SAMP is associated with signs of advanced senescence, such as reduced activity, hair loss, lack of hair glossiness, skin coarseness, periophthalmic lesions, increased lordokyphosis of the spine, and a shortened life span. In the SAMP series, SAMP8 showed more spontaneous age-related deterioration of memory and learning abilities in passive avoidance response tests (Miyamoto et al. 1986), especially in the acquisition stage (Yagi et al. 1988), than did control SAMR1 mice.

Shimada (1999) reviewed age-dependent cerebral atrophy and cognitive dysfunction in SAMP10 mice.

Banks et al. (2003) found an impairment of efflux of human and mouse amyloid β proteins 1–40 and 1–42 from brain in SAMP8 mice.

Farr et al. (2004) reported that dehydroepiandrosterone sulfate improved learning and memory in aged SAMP8 mice.

Epileptic fowl having a hereditary form of primary generalized epilepsy are characterized by tonic-clonic seizures (Gervais-Fagnou and Tuckek 1996).

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F.4.4

Genetically Modified Animals

The use of genetically altered animals in biological research has also affected research in learning

and memory. Genetically engineered strains of mice, modified by transgenesis or gene targeting (“knock-out”) have been generated and are used as research tools for deciphering the genetic basis of behavior. These animals may be helpful to evaluate the efficacy of new pharmacological treatments (Mayford et al. 1997; Costentin 1998; Anagnostopoulos et al. 2001; Higgins and Jacobsen 2003). Various authors described transgenic mouse models of Alzheimer’s disease (van Leuven 2000; Götz 2001; Janus and Westaway 2001).

Several strains were described, such as:

Transgenic mice, which **overexpress S100 β** , a calcium binding astrocytic protein influencing hippocampal long-term potentiation (LTP) and depression (LTD) (Roder et al. 1996),

Transgenic **Cu/Zn-SOD mice overexpressing the gene encoding copper/zinc superoxide dismutase** which is also overexpressed in human Down syndrome (Gahtan et al. 1998),

a mutant mice strain exhibiting delayed Wallerian degeneration (Fox and Faden 1998),

Mice with a **modified β -amyloid precursor protein gene** (Tremml et al. 1998; Kim et al. 2004),

Gozes et al. (1993) reported learning impairment and prolonged retardation in memory acquisition in transgenic mice carrying a chimeric gene for the vasoactive intestinal peptide (VIP).

Franowicz et al. (2002) compared mice with a point mutation of the α_{2A} -adrenoceptor, which serves a functional knockout with wild mice. Working memory performance was impaired and the cognitive enhancement by guanfacine was lost.

Silva et al. (1992) studied memory mechanisms in mutant mice that do not express the alpha-calcium-calmodulin kinase II. These mice are deficient to produce hippocampal long-term potentiation and were recommended as a suitable model for studying the relation between long-term potentiation and the learning process.

Moechars et al. (1999) reported early phenotypic changes in transgenic mice cloned by cDNA coding of human wild-type **APP (695 isoform)**, the Swedish (K670N,M671L) mutant (770 isoform) and the London (V642I) mutant (695 isoform) in the pTSC vector in the mouse *thy-1* gene (Moechars et al. 1996).

Strazielle et al. (2003) investigated regional brain cytochrome oxidase activity in β -amyloid precursor protein transgenic mice with the Swedish mutation. **APP23 mice** were generated under the control of the murine Thy-1 gene promoter and were backcrossed by several generations to the C57BL6J background

(Sturchler Pierrat et al. 1997; Phinney et al. 1999; Boncristiano et al. 2002).

Using this model, Kelley et al. (2003) reported progressive age-related impairment of cognitive behavior in APP23 transgenic mice.

Van Dam et al. (2005) described the symptomatic effects of donepezil, rivastigmine, galantamine and memantine of cognitive deficits in the APP23 model.

Johnson-Wood et al. (1997) studied amyloid precursor protein processing and A β_{42} deposition in the PDAPP transgenic mouse model of Alzheimer’s disease.

Touma et al. (2004) investigated the TgCRND8 line, a double mutant (Swedish and Indiana) human APP₆₉₅ model of Alzheimer’s disease, generated by Chishti et al. (2001).

Steiner et al. (2001) found that **galanin transgenic mice** display cognitive and neurochemical deficits characteristic of Alzheimer’s disease. The mouse galanin gene was coupled to the human dopamine β -hydroxylase reporter (Mazarati et al. 2000; Cadd et al. 1992).

Huber et al. (2002) and Qin et al. (2002) described a **fragile X knockout mouse (*fmr1*KO)** mouse, which resembles the fragile X mental retardation syndrome in humans.

Richardson et al. (2003) described a transgenic mouse line overexpressing the 695-amino-acid isoforms of human amyloid precursor protein harboring the Swedish double familial Alzheimer’s disease mutation. This line, referred as **TAS10**, exhibits neuropathological features and cognitive deficits that are closely related to the accumulation of A β in their brain and that are reminiscent to those observed in Alzheimer’s disease.

Oddo et al. (2003) generated a triple transgenic model of Alzheimer’s disease harboring three mutant genes: **β -amyloid precursor protein (β APP_{Swe})**, **presenilin-1 (PS1_{M146V})**, and **tau P_{301L}**. The mice progressively develop A β and tau pathology, with a temporal- and regional-specific profile that closely mimics their development in the human Alzheimer’s disease brain.

The **Tg2576 transgenic model** of Alzheimer’s disease was studied by several authors (Hsiao et al. 1996; Haugabook et al. 2001; King and Arendash 2002; Lehman et al. 2003; Li et al. 2003; Parvathani et al. 2003; Quinn et al. 2003; Zong et al. 2003; Middei et al. 2004; Rodrigo et al. 2004). Tg2576 transgenic mice overexpressing a mutant gene for β -amyloid precursor protein develop A β deposits at the age of 9–11 months. At this time, the animals are impaired in Y-maze spon-

taneous alteration, visible platform recognition, and several sensorimotor tasks. Gong et al. (2004) tested immunization of Alzheimer model (Tg2576) mice with adenovirus vectors encoding amyloid β -protein and granulocyte-macrophage colony-stimulating factor (GM-CSF) and found a reduction of amyloid load in the brain.

Skovronsky et al. (2000) synthesized a fluorescent ligand, [(*trans,trans*)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)-styryl]benzene (BSB)] which allows *in vivo* detection of amyloid plaques in the Tg2576 transgenic mouse model of Alzheimer's disease.

Tanemura et al. (2001, 2002) and Tatebayashi et al. (2002) generated **Tg mouse lines expressing R406W human tau**.

Reilly et al. (2003) analyzed amyloid deposition in the hippocampus and entorhinal cortex in a transgenic mouse model. Heterozygous **PDAPP transgenic mice** carrying the APPV717F familial AD mutation (Murrell et al. 1991) were bred from the established line PDAPP-109 over several generations on hybrid background representing combinations of C57BL/6. DBNA, and Swiss-Webster strains (Games et al. 1995).

Using these mice strains, Rockenstein et al. (1995) investigated levels and alternative splicing of amyloid β protein precursor (APP) transcripts in brains of APP transgenic mice.

Postina et al. (2004) generated transgenic **mice overexpressing either ADAM10** or a catalytically inactive mutant. ADAM (adisintegrin and metalloprotease) 10 has many properties of a physiologically relevant α -secretase: it cleaves APP-derived peptides at the main α -secretase cleavage site between positions 16 and 17 of the A β region and is expressed in mouse and human brain (Hartmann et al. 2002). A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer's disease mouse model.

Apolipoprotein E plays a major role in the amyloid cascade. Xu et al. (1999), Levi et al. (2003), Ophir et al. (2003) and Dolev and Michaelson (2004) used **apoE3- and apoE4-transgenic mice** for their studies.

Oike et al. (1999) generated a mouse model of the **Rubinstein-Taybi syndrome** by an insertional mutation into the cyclic AMP response element-binding protein gene. These mice showed deficiencies in long-term memory and other clinical features of the Rubinstein-Taybi syndrome, such as growth retardation, retarded osseous maturation, hypoplastic maxilla, cardiac anomalies, and skeletal abnormalities.

Impairment of the ubiquitin/proteasome system has been proposed to play a role in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Lindsten et al. (2003) developed a model for *in vivo* analysis of the **ubiquitin/proteasome system** by generating mouse strains transgenic for a green fluorescent protein reporter carrying a constitutively active degradation signal.

Higuchi et al. (2005) generated transgenic **mice that overexpress human calpastatin**, a specific and the only natural inhibitor of calpains. Distinct mechanistic roles of calpain and caspase activation in neurodegeneration were revealed in these mice.

Hoyle et al. (2006) reported impaired performance of **alpha7 nicotinic receptor knockout mice** in the five-choice serial reaction time task.

Doubly transgenic mice have been used by several authors to analyze Alzheimer-like pathology (Arendash et al. 2001; Battaglia et al. 2003; Gong et al. 2004; Trinchese et al. 2004).

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F.4.5

Studies in Invertebrate Animals

F.4.5.1

Studies in *Caenorhabditis Elegans*

The worm *Caenorhabditis elegans* has been recommended as a model of human disease (Baumeister 2002). Link (2001, 2005) reviewed invertebrate models of Alzheimer's disease with special emphasis on *Caenorhabditis elegans*.

Link et al. (2001) reported visualization of fibrillar amyloid deposits in living, transgenic *Caenorhabditis elegans* animals using the sensitive amyloid dye X-34.

Drake et al. (2003) found that oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid β -peptide (1–42) in a transgenic *Caenorhabditis elegans* model.

Link et al. (2003) described gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. Transgenic *Caenorhabditis elegans* animals were engineered to inducibly express the human beta amyloid peptide.

Strayer et al. (2003) reported that expression of the small heat-shock protein Hsp16–2 in *Caenorhabditis elegans* is suppressed by *Ginkgo biloba* extract EGb 761.

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F.4.5.2

Studies in *Drosophila*

Götz et al. (2004) reviewed transgenic animal models of Alzheimer's disease and related disorders mentioning mice, fish (*Petromyzon marinus*, the sea lamprey), worms (*Caenorhabditis elegans*), and flies (*Drosophila melanogaster*).

Ferber (2001) recommended using the fruit fly to model tau malfunction.

Shulman and Feany (2003) conducted a genetic modifier screen in a *Drosophila* model of tauopathy to investigate the molecular mechanisms responsible for Tau-induced neurodegeneration.

Finelli et al. (2004) showed that overexpression of A β 42 peptides in the nervous system of *Drosophila melanogaster* results in phenotypes associated with neuronal degeneration in a dose- and age-dependent manner.

Crowther et al. (2004) reported that transgenic flies expressing the toxic β -amyloid develop neurodegeneration. The flies exhibit a clear phenotype from a few days of age, including reduced locomotor function, impaired olfactory memory and shortened lifespan. Therapeutic agents that interfere with the generation of toxic aggregates of β -amyloid peptides have been shown to rescue the flies. *Drosophila* is recommended as a model of Alzheimer's disease.

Iijima et al. (2004) used the *Drosophila* model to compare the specific pathological roles of human A β 40 and A β 42 in Alzheimer's disease.

Nichols (2006) published a survey on *Drosophila melanogaster* neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery.

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Chapter G

Effects On Peripheral Nerve Function

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G.1.1

Conduction Anesthesia

G.1.1.1

Conduction Anesthesia in the Sciatic Nerve of the Frog

PURPOSE AND RATIONALE

Based on earlier studies by Sollmann (1918), Fromherz (1922), Fußgänger and Schaumann (1931), and Bülbring and Wajda (1945) on plexus anesthesia in frogs, Ther (1953a, b, 1958) described a method for conduction anesthesia of the sciatic nerve in frogs.

PROCEDURE

Frogs (*Rana temporaria*) of either sex are used and are kept at 4°C. The frog is decapitated with a pair of scissors. The skin is incised in the thigh region at both sides and the sciatic nerves are carefully exposed in the thigh, avoiding any stretching and injury of the nerve. The frog is suspended on a vertical board. Small pieces of white cotton are soaked with different concentrations of the test preparations (between 0.05% and 1%) or the standard and placed gently around the sciatic nerve for 1 min. Then the cotton swab is removed and the frog is placed with its extremities into a bath with 0.65% NaCl solution. This allows testing for duration and reversibility of the local anesthetic effect. One side is used for the test preparation and the other for the standard (e. g., 0.25% butanilcaine). Every 3 min the frog is removed from the bath and the toes of the legs or the ankle joint are pinched three times with a small forceps. The reflex contraction is abolished when conduction anesthesia is effective. The stimuli are repeated every 3 min until anesthesia vanishes. Two to 5 frogs are used for each concentration.

EVALUATION

Time of onset and duration of anesthesia are recorded for each concentration. Time-response and dose-response curves can be established.

MODIFICATIONS OF THE METHOD

In the original method by Bülbring and Wajda (1945) the frogs were decapitated and the upper part of the spinal cord was destroyed down to the level of the third vertebra. The viscera are removed exposing the lumbar plexus without damaging it. The frog was pinned to a vertical board and the solution of the local anesthetic dissolved in 0.7% saline is put into the pocket formed by the lower abdomen. Different concentrations of HCl are used as stimuli into which the feet of the frog were immersed every min.

Idänpään-Heikkilä and Guilbaud (1999) used a rat model of trigeminal neuropathic pain where the neuropathy is produced by a chronic constriction injury of the infra-orbital branch of the trigeminal nerve, and studied the effects of various drugs on this purely sensory model of neuropathic pain (see H.1.2.11)

For mechanical stimulation, a graded series of ten of von Frey filaments with a bending force between 0.217 and 12.5 g was used. The stimuli were applied within the infra-orbital nerve territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. Local injection of an local anesthetic (articaine) into the rostral orbital cavity of the lesioned side, into the close proximity of the ligated infra-orbital nerve increased the mechanical threshold to the upper level. The duration of the effect was dose-dependent.

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G.1.1.2

Conduction Anesthesia in the Sciatic Nerve of the Rat

PURPOSE AND RATIONALE

Based on earlier studies (Truant 1958; Truant and Wiedling 1958/59, Åström and Persson 1961) Camougis and Takman (1971) have written a detailed description for testing conduction anesthesia of the sciatic nerve in the rat.

PROCEDURE

Male Wistar or Sprague Dawley rats weighing 125 to 175 g are used. The animal is suspended in a prone position by grasping the base of the tail and thoracic cage. A hind limb is extended to its full length and the depression for needle insertion is located by palpation with the left index finger. The site of injection is the area under the skin at the junction of the biceps femoris and the gluteus maximus muscles. The sciatic nerve is blocked in the midthigh region with 0.2 ml of the drug solution administered by a 24- to 25-gauge needle attached to a 0.25 ml tuberculin syringe. Usually a 1% solution of the test drug in 0.9% NaCl is used as a test solution. The other leg is used for a control drug (e. g., procaine or lidocaine). Immediately after the injection, repeated checks of the digit of the foot and the walking behavior are performed. In the normal foot, the digits are wide apart, while in the blocked leg the digits of the foot are close together. Also the successful block is evidenced by dragging of the leg and an inability of the animal to use the leg in walking up the inclined wire mesh cover of the cage. After the time of block for each leg is noted, each animal is examined every 5 to 10 min in order to note the time of recovery.

EVALUATION

From the data, averages for onset and duration of action are calculated, plus the frequency of blocks are

noted. Using various doses of test compound and standard, dose-response curves can be established and potency ratios calculated.

MODIFICATIONS OF THE METHOD

With a similar technique Lembeck (1953) tested the effect of the added vasoconstrictive agents adrenaline, noradrenaline and Corbasil to procaine.

Sciatic nerve blockade in the **rat** was used by Feldman and Covino (1988) to study comparative motor-blocking effects of bupivacaine and ropivacaine.

Grant et al. (1992) used a **rat** sciatic nerve model for independent assessment of sensory and motor block induced by local anesthetics. Motor block was assessed by measuring hind paw grip strength with a dynamometer. Sensory block was determined by measuring hind paw withdrawal latency from radiant heat.

Kohane et al. (1998) studied sciatic nerve blockade with ropivacaine and bupivacaine in infant, adolescent, and adult **rats**. Rats were anesthetized briefly with halothane by face mask. The sciatic block was conducted by introducing a 30-gauge needle posteromedially to the greater trochanter, pointing in an anteromedial direction. Once bone was contacted, the needle was withdrawn 1 mm and drug was injected. Nerve blockade was assessed by withdrawal of the hind leg after pinching the lateral aspect of the plantar surface, by a modified hot-plate test, by the positional placing response as a test of proprioception, by extensor postural thrust as a measure of motor strength, and by ability to hop. Thermal latency in the contralateral (uninjected) limb was determined as a measure of systemic toxicity.

Grant et al. (2001) tested perineural antinociceptive effects of opioids in a **rat** model. Analgesia was assessed using the hind paw latency response to radiant heat.

Luduena and Hoppe (1952) used **guinea pigs** to test sciatic nerve block by local anesthetics. The anesthetic solutions were injected close to the sciatic nerve. Sensory paralysis was determined by pricking the posterior site of the thigh with a needle. The effect on motor nerve fibers was determined by observing the abnormal position of the injected leg in locomotion.

Siems and Soehring (1952) described a model in **guinea pigs** resembling peridural and paravertebral anesthesia in man.

Åkerman et al. (1988) used **guinea pigs** to determine brachial plexus block. The syringe was directed towards a line between the head of the humerus and the manubrium of the sternum and the first ribs. The

needle was inserted about 1.5 cm into the pocket felt as a depression by palpation between the head of the humerus and sternum and associated structures. Following retraction of the needle by a few millimeters, 0.2 ml of the solution was injected. The orientation of the needle was changed 3–4 times during the injection to enable all branches of the plexus to be reached. Assessment was made of motor and sensory blockade.

Leszczynska and Kau (1992) used a sciatic nerve blockade method in **mice** to differentiate drug-induced local anesthesia from neuromuscular blockade. The drugs were injected into the popliteal space of the right hind-limb. A positive local anesthetic activity was recorded when a mouse was only able to walk using three limbs on an inverted wire mesh screen and the injected limb was hanging in the air. A positive neuromuscular blockade was recorded when a mouse could walk normally on the top of the wire mesh screen but was unable to stay on the inverted screen.

Thut et al. (1995) used the **rabbit** tooth-pulp assay (see H.1.2.8) to quantify efficacy and duration of antinociception by local anesthetics infiltrated into maxillary tissues.

Rosenberg and Heinonen (1983) found a differential sensitivity of A and C nerve fibers to long-acting local anesthetics.

The Local Anesthetics for Neuralgia Study Group (1994) designed a surgically implantable nerve irrigation system for intermittent delivery of local anesthetics and evaluated long-term performance and histocompatibility in rats.

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G.1.1.3

Conduction Anesthesia on the Mouse Tail

PURPOSE AND RATIONALE

The radiant heat method as being used for evaluation of systemic analgesic activity (D'Armour et al. 1941; Ther et al. 1963; Grant et al. 1993) can also be used for determination of conduction anesthesia by injecting the local anesthetic into the root of the tail.

PROCEDURE

Groups of 10 mice (NMRI-strain) of both sexes with a weight between 18 and 22 g are used for each dose. Before administration of the test compound or the standard the normal reaction time is determined. The animal is placed into a small cage with an opening for the tail at the rear wall. The tail is held gently by the investigator. By opening of a shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. After about 6 s, the reaction of the animal is

observed by the investigator. The mouse tries to pull the tail away and turns the head. The shutter is closed with a switch when the investigator notices this reaction. Mice with a reaction time of more than 6 s are not used in the test.

The test compounds and the standard are injected in a volume of 0.1 ml on both sides in the area of the tail root. The animals are submitted to the radiant heat again after 10 min. The area of heating is about 1.5 cm distal to the injection site. For each individual animal the reaction time is noted.

EVALUATION

There are two possibilities for evaluation:

1. The average values of reaction time after each time interval are calculated and compared with the pretest value by analysis of significance.
2. At each time interval only those animals which show a reaction time twice as high or higher as the pretest value are regarded as positive. Percentages of positive animals are counted for each time interval and each dose and ED_{50} values are calculated according to LITCHFIELD and WILCOXON.

MODIFICATIONS OF THE METHOD

Bianchi (1956) used the tail-clip method of Haffner (1929) as a simple quantitative method for testing local anesthetics. Fully grown albino **mice** of either sex were used. A small artery clip with its blades covered by thin rubber tube was applied to the root of the tail. Those animals that did not show the pain reflex (the mice turn again and again trying to remove the clip) within 30 s were eliminated. The remainder received subcutaneously, about 1 cm from the root of the tail, 0.1 ml of the solution of drug. The pain reflex is tested 15 min after injection, applying the stimulus to the zone where the compound was injected. The proportion of animals that proved to be anesthetized was noted for each dose.

Saxen et al. (1993) and Smith (1997) described the mouse paw withdrawal assay: a method for determining the potency and duration of the effect of local anesthetics in mice. A standard rodent tail-flick apparatus was used to stimulate the dorsal skin of each hind paw in the mouse. The radiant heat intensity of the lamp was adjusted to yield hind paw withdrawal latencies of 6–8 s, with a 20-s cut-off time to prevent tissue damage. Testing was performed by gently restraining the animal in a small towel and passively placing one hind paw on the stage so that the animal was free to withdraw its paw from the stimulus without restric-

tion. Paws were tested in alternative order (right, then left; left, the right, etc.) to minimize procedural bias. The individual left and right paw withdrawal latency times were determined in each mouse and the difference between left and right latencies were calculated. Paw withdrawal time difference was calculated by subtracting the experimentally modified paw withdrawal latency from the control paw latency.

Grant et al. (1993) quantified the duration of the local anesthetic-induced conduction block in the mouse using the tail flick test.

Grant et al. (1994) studied prolonged analgesia with liposomal bupivacaine in mice using the tail flick test.

The tail flick procedure using **rats** as test animals has been proposed by Herr et al. (1953).

Madan et al. (1970) determined conduction anesthesia by a tail-pinch technique in rats. The local anesthetic was injected subcutaneously bilaterally at the root of the tail. The test for local anesthesia was begun 15 min after injection by pinching with a polyethylene sheathed artery forceps and repeated every 15 min. The number of animals failing to remove the forceps by biting was recorded.

Kamerling et al. (1985) described a method for studying cutaneous pain perception and analgesia in **horses**. This method was used by Harkins et al. (1996, 1997) for determination of highest no effect dose (HNED) for local anesthetic responses to procaine, cocaine, bupivacaine, benzocaine and of a plant extract. A heat projection lamp was used as noxious stimulus directed onto the pastern to elicit a flexion-withdrawal reflex. Hoof withdrawal latency was defined as the time between lamp illumination and withdrawal of the hoof. The local anesthetic drugs were injected into the area of the palmar nerve where it passes lateral to the sesamoid bone.

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G.1.1.4

Rabbit Tooth Pulp Assay

PURPOSE AND RATIONALE

The rabbit tooth-pulp assay (see H.1.2.8) has been used successfully to evaluate the potency, efficacy, and duration of antinociception of analgesics (Yim et al. 1955; Piercey and Schroeder 1980; Wynn et al. 1984). Thut et al. (1995) described a method for administration of local anesthetic drugs to the maxillary arch of rabbits and subsequent measurement of antinociceptive action.

PROCEDURE

Pulp chambers are exposed close to the facial gingival line of the two central incisors in New Zealand White male rabbits (1.5–2.0 kg) using a high-speed dental drill and 0.5-mm round burr immediately after the i.v. administration of 30 mg/kg ketamine. All experiments are conducted 48 h or more after pulp exposure. A linear ramp function stimulator is used as the direct current voltage source. The rabbit is slightly restrained, and the current is applied to the pulp via fine-wire platinum electrodes held in each of the cavities. Linearly rising voltage is applied at a rate of 0.33 V/s until

a patterned lick-chew response occurs. Mean threshold voltages are established for controls using an average of 3 determinations, and then obtained after treatment using a single determination up to a maximum of 10 V. Rabbits having control values greater than 3 V are excluded from the study. The injection site of the test drug is 1.5 cm posterior to the central incisors and 4 mm below the roof of the maxillary buccal vestibule. The buccal vestibule is the area where the cheek and the top of the maxillary tissue join. The tip of a 27-Ga × 1/2" syringe needle is inserted until it contacts the cancellous bone. The volume of injection is 0.4 ml. Injections are made bilaterally so that the total volume injected is 0.8 ml. The animals can be used chronically, but no rabbit should be exposed to the same drug or the same dose more than once, and non should be injected more frequently than very third day. One animal is used per dosage when analgesia is observed to be either 0% or 100%. Two or 3 animals are used for dosages with effects between 0% and 100%. When more than one animal is used, data are averaged and treated as a single observation. Pulp chambers have to be re-exposed when the drilled openings disappear because of incisor growth (approximately after 3 weeks).

EVALUATION

The percentage of maximum effect (*MPE*) for each observation time is calculated from the following equation:

$$MPE = (TV - CV)/(IOV - CV) \times 100$$

TV is the voltage after treatment, *CV* is the control voltage, and *IOV* are the maximum volts applied. The voltage that elicits the response is recorded at zero time and 5, 10, 15, 30, 60, 120, and 180 min after injection. The *MPE* is calculated for each observation time. Graphs of the *MPE* values versus time are constructed, and the maximal effect and time of the maximal effect are recorded. Further, the duration of each dosage is defined as the length of time that that dose achieved a *MPE* greater than 25%. Data are regressed between observed time points to provide estimates of the time when 25% *MPE* is first achieved and also the time when 25% *MPE* is no longer observed. A computer program or the standard method of Litchfield and Wilcoxon (1949) can be used to calculate *ED*₅₀, *ED*₉₀, and *ED*₉₅ values with 95% confidence limits.

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G.1.1.5

Infraorbital Nerve Block in the Rat

PURPOSE AND RATIONALE

Several authors have used the infraorbital nerve block in the rat to study the potency and duration of action of local anesthetics (Fink et al. 1975, 1978; Ready and Fink 1980; Buckley and Fink 1981; Buckley et al. 1985; Renck et al. 1988; Hassan et al. 1989, 1993).

PROCEDURE

Male Sprague-Dawley rats weighing 500–600 g are used. The infraorbital nerve of the rat is 2–3 mm in diameter and supplies the upper lip and whisker area. It is homologous with the infraorbital nerve in humans. Since the rat lacks a closed orbit, the nerve runs forward beneath the eye and emerges from the maxilla through a deep notch rather than a foramen. The animals are lightly anesthetized with intraperitoneal phenobarbitone 25 mg/kg. This degree of sedation abolishes the righting reflex but does not interfere with the generalized aversive response to pinching the upper lip with an artery forceps. Test solution (0.2 ml) is injected unilaterally on the right side. The intact left side serves as control for comparing the responses on the two sides. Only animals whose aversive response is abolished within 60 s of the time of injection are included in the study. The animals are tested at 5-min intervals thereafter until the first sign of return of the aversive response on the injected side. Ten animals are used for each concentration of each test compound.

EVALUATION

The incidence of blocks at each concentration is recorded. The duration of the analgesic block (\pm SD) is calculated for each concentration and each test compound.

MODIFICATIONS OF THE METHOD

Renck and Hassan (1992) tested analgesia of the upper lip by electrical stimulation using externally applied bipolar stimulatory electrodes (blunted 27-gauge hypodermic needles) connected to a constant current stimulator delivering one impulse per second of 0.24 ms duration.

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G.1.1.6

Retrobulbar Block in Dogs

PURPOSE AND RATIONALE

Defalque and Stoelting (1976) published a method to determine latency and duration of action of local anesthetics after retrobulbar injection in dogs.

PROCEDURE

Young female mongrel dogs weighing 13–15 kg are used. Twenty-four h before the test, 0.25% eserine ointment is placed in each conjunctival sac of the dog. Pentobarbital (25 mg/kg) is administered intravenously, then repeated with 10 mg/kg at hourly intervals, thus maintaining the animal in light anesthesia (corneal reflex present). Ten min after induction, the dog is put into 30-degree head-down position, and 20 ml of 0.05% tetracaine is forced into the epidural

space through the interarcuate ligament. Horner's syndrome occurs within 5 min.

A 150-watt surgical lamp is now focused upon the eye from an 1 meter distance. Fifteen min later, a retrobulbar block is performed: The sclera is seized with an ophthalmic forceps and the eyeball is pulled downward and medially; a 23-gauge needle is then introduced through the superior rectus muscle, tangentially to the globe, and is immobilized as soon as a click indicates penetration of the retrobulbar space; correct placement is confirmed by free motion of the needle tip and protrusion-rotation of the eyeball upon injection of 1 ml of air.

After aspiration, 2 ml of the tested anesthetic is then injected at a rate of 0.5 ml per second. The pupil dilates and reaches its maximal diameter (6 mm) within a few minutes. This apparent diameter is estimated with a 2-cm long ruler calibrated in millimeters, whose center is gently applied to the corneal center. The pupil is measured every 15 s for 5 min, then every 5 min until reappearance of maximal miosis (pinpoint and asymmetrical), a precise endpoint which generally coincides with corneal reflex and lacrimation.

EVALUATION

Drug latency (in min) and duration (in 5-min units) are averaged for both eyes of each animal, and the mean and standard deviation then calculated for all test animals. Analysis of variance is performed to find significant differences between various local anesthetics.

REFERENCES AND FURTHER READING

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G.1.1.7

Isolated Sciatic Nerve Preparation of the Frog

PURPOSE AND RATIONALE

Isolated nerves are immersed between stimulating and recording electrodes in solutions containing local anesthetics which allows electrophysiological measurements (Paterson and Hamilton 1970).

PROCEDURE

A large frog is sacrificed by decapitation with a pair of scissors, and the spinal cord severed. The skin around the entire body in the region immediately posterior to the forelimbs is cut, and the abdomen and hind legs are skinned by peeling the skin in a posterior direction. The fat, muscles, and other tissues are cut

at the posterior end of the body so as to expose the nerves extending posteriorly from the cord. The muscles of the thigh are cut and retracted so as to expose the sciatic nerve. Finally, the gastrocnemius muscle is removed and the peroneus muscle is extended laterally from the tibiofibula. The entire sciatic nerve with branches should now be exposed. A thread with a loop next to the nerve is tied around the nerve in the region of the sacral vertebra. The nerve is then cut anterior to the thread. During the isolation procedure the nerve is kept moist with frog Ringer solution. The connective tissue and the nerve branches are cut carefully. The nerve is finally cut near the distal end of the tibiofibula and extended on a filter paper previously soaked with Ringer solution. With the nerve anchored by the thread, the remaining connective tissue is peeled back toward the distal end of the nerve. Any remaining branches are cut at the point of their bifurcation. The distal end of the nerve is tied with a length of thread. The sciatic-peroneal nerve preparation is mounted in a nerve chamber for recording.

Electrodes for stimulation are placed on the proximal end of the nerve while recording electrodes are placed at the distal end. The nerve is immersed in a trough containing frog Ringer solution as bathing fluid. The stimuli are applied with a commercial stimulator (e. g., Grass Model S4) with a duration of 5 ms, a frequency between 30/s, and 1.5 to 15 mV. The recording electrodes are connected with an amplification and display system. Stimulation of the nerve produces a display on the oscilloscope. Adjusting the voltage setting on the stimulator will cause the action potential to increase and decrease correspondingly on the scope. After pinching the nerve between the last two recording sites, the action potential should be monophasic and appear entirely above the base line. The spike is observed periodically for several min to ascertain its stability.

The bathing trough is changed to a trough containing the local anesthetic dissolved in Ringer solution. At one or 2 min intervals the amplitude of the spike is read and recorded on a polygraph. After 5 min treatment, the drug trough is removed and the nerve is immersed in the Ringer solution of the bathing trough. Amplitudes are measured in periodic intervals and thus the recovery process is recorded.

EVALUATION

Block and recovery curves are achieved with various concentrations of the test compound and the standard. Dose-response curves can be obtained.

MODIFICATIONS OF THE METHOD

Specialized techniques with isolated nerves (desheathed nerves, equilibrium blocks, single nerve fibers) have been described by Camougis and Takman (1971).

Den Hertog (1974) used the **desheathed cervical vagus of the rabbit** mounted in a single sucrose gap apparatus and measured changes in the membrane potential and compound action potential. The ratio of the distance between stimulation and recording electrode (15 mm) and the time between stimulation of the nerve and the top of the action potential was taken as an index for the mean conduction velocity.

Salako et al. (1976) tested a new anti-arrhythmic drug as a local anesthetic on desheathed **frog** nerve.

Lee-Son et al. (1992) used the sucrose-gap method on desheathed isolated frog peripheral nerves to study the stereoselective inhibition of neuronal sodium channels by local anesthetics.

Lambert et al. (1994) studied the reversibility of conduction blockade in desheathed bullfrog sciatic nerves, using the sucrose gap method for recording compound action potentials, before and during exposure to local anesthetics and during drug washout.

Isolated nerves from other species have been used for the study of local anesthetics, such as those from rabbits by Ritchie et al. (1965a, b), or from rats by Condouris and Lagomarsino (1966), Štolc and Mai (1993).

Gissen et al. (1980) observed differential sensitivities of mammalian nerve fibers to local anesthetic agents in rabbits. A fibers were blocked at the lowest drug concentrations, the intermediate B fibers were blocked at a higher drug concentration, and the smallest, slowest-conducting C fibers required the highest drug concentration for conduction blockade.

Fink and Cairns (1984) showed differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons in rabbits.

Åkerman et al. (1988) studied the block of evoked action potential in the isolated sheathed sciatic nerve of the frog for primary evaluation of a local anesthetic compound.

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G.1.1.8

Isolated Mammalian Sciatic Nerve Preparation

PURPOSE AND RATIONALE

Karlsson et al. (1994) tested the local anesthetic-like effects of an NK₁ receptor antagonist in a mammalian sciatic nerve preparation using guinea pigs.

PROCEDURE

Male guinea pigs weighing 250–400 are sacrificed and the sciatic-tibial nerves immediately excised and cleaned in buffer equilibrated with 95% O₂ + 5% CO₂ under a dissecting microscope. The nerve is mounted in a Plexiglas chamber consisting of three connecting wells. The central well is perfused with buffer at a rate of 5 ml/min and contained the grounding electrode (Ag/AgCl), the in- and outlet of the peristaltic pump driven perfusion system and a thermistor probe. The two lateral wells (one for stimulating, the other for recording) are filled with mineral oil. Stimulating and recording electrodes are made from bare platinum-iridium wire. The passages between the central and lateral wells are sealed with petroleum jelly. The length of the nerve exposed to the drug in the central well is 8 mm. The nerve chamber is kept at a temperature of $27.0 \pm 0.5^\circ\text{C}$.

A constant current supramaximal stimulus of 50 μs duration is used throughout the experiment. The intensity of the stimulus is ten times greater than the threshold intensity required to elicit a compound action potential. Throughout the experiment the nerve is stimulated at the low frequency of 1/min to determine basal compound action potential amplitude. Once the response has stabilized, a single burst of pulses (pulse train duration 250 ms, at 40 Hz) is applied approximately 5 min before drug application (control train) and again after drug application to determine the extent of frequency dependent block. The compound action potentials are digitized with a computer interface and a software program and recorded on a PC. A two channel storage oscilloscope is used to follow the experiment and to automatically sample and calculate the amplitudes of the compound action potentials.

The drugs are dissolved in buffer and applied approximately 5 min after the control train. The development of basal block following drug application is followed with 0.0167 Hz stimulation. In the case of lidocaine, the maximal inhibition is reached 20–30 min after application. The basal block is allowed to reach equilibrium, which is defined as the condition in which the difference in amplitude between the first and the last of five consecutive responses is less than 1% of the control compound action potential amplitude obtained prior to drug application. To assess frequency dependent block, a second stimulus is given after equilibrium is reached. Once basal and frequency dependent block has been determined, the nerves are washed with fresh buffer.

EVALUATION

The magnitude of the basal block (%) is calculated by comparing the average peak amplitude of the last three compound action potentials with the average peak amplitude of the last three compound action potentials prior to drug application. Similarly, frequency dependent block is expressed in percent of the control train, and is effectively the basal block plus the additional decrease in compound action potential amplitude. Dose-response curves for the standard and the test drugs are constructed by plotting basal block and frequency dependent block against drug concentration. The pIC_{50} values (the negative logarithm of the molar concentration of the drug producing 50% inhibition of the compound action potential amplitude) are estimated by linear regression analysis of the results in the 10–90% interval of the dose-response curves.

The dose-response data obtained with the standard and the test drugs and the effect of a single concentration (0.5 mM) of all compounds on basal block and frequency dependent block are analyzed by ANOVA. The values of latency to the peak of compound action potential are analyzed by paired *t*-test.

MODIFICATIONS OF THE METHOD

Fink and Cairns (1984) studied differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons.

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G.1.1.9

Effect of Local Anesthetics on Different Nerve Fibers

PURPOSE AND RATIONALE

Wildsmith et al. (1989) examined the *in vitro* sensitivities of different types of fibers in rabbit vagus nerves to

local anesthetic block with a range of local anesthetic drugs at high and low frequency stimulation. Rapidly conducting, myelinated (A) fibers are more sensitive than more slowly conducting, unmyelinated (C) fibers.

PROCEDURE

The cervical portions of the vagus nerves of rabbits weighing 2.5 kg are removed, desheathed and mounted in an airtight chamber which is maintained at 37°C by a water jacket. A central 1-cm section of the nerve chamber is perfused at 0.5 ml/min with carbonated Liley solution (pH 7.4 ± 0.02 after equilibration with 5% carbon dioxide in oxygen). A tap system allows the perfusate to be changed to test solution without admission of air.

A square wave generator is used to apply supramaximal electrical stimuli (10–15 V for 1 ms) at a rate of 0.0167 Hz during a 30-min period of stabilization. Two trains of stimuli (duration 0.25 s) are applied, one at 20 Hz and the other at 40 Hz. The preparation is considered valid only if there is less than 5% decrement in height of the action potential during stimulation at 40 Hz. The signals from the nerve are amplified, digitized (Unilab 532.001 interface sampling at 40 μ s intervals for 8 ms, then at 200 μ s intervals for 50 ms) and recorded with a microcomputer based system. The numeric derivative of the signal can be printed out immediately or stored on disc for subsequent processing. Signals are monitored also on a storage oscilloscope.

A submaximal blocking concentration of the hydrochloride salt of a test drug dissolved in carbonated Liley solution is applied. Only one concentration of one drug is applied to each nerve. The stimulation rate remains at 0.0167 Hz until any changes in the compound action potential are complete (minimum period 30 min), when the trains of high frequency stimulation are repeated. Drug effect is measured as the percent decreases in the height of the three components (A, B and C fibers) of the compound action at each frequency of stimulation. At 20 and 40 Hz, the last signal produced by each train of stimulation is used for analysis. An experiment is considered valid only if the action potentials recover to more than 90% of control height on washing the drug from the nerve.

EVALUATION

From the data obtained, the ED_{50} (and its SE) for the effect of each drug on each fiber type at each rate of stimulation is determined by log-probit analysis. Plots of the development of block while the nerves are being stimulated at 0.0167 Hz are also made. From these an index of the rate of development of A and C fiber block

is derived ($T_{1/2B}$). This is defined as the time taken to develop 50% of the eventual maximum degree of block for the particular fiber type in each experiment.

MODIFICATIONS OF THE METHOD

Scurlock et al. (1975) examined the relative sensitivity of sympathetic preganglionic and postganglionic axons, B and C fibers, respectively, to structurally dissimilar local anesthetics. Cervical sympathetic trunks from adult rabbits were submerged in Krebs-Henseleit solution except during the brief recording periods, when they were stimulated electrically with twice maximal square-wave pulses of 0.15 ms (B fibers) or 0.5 ms (C fibers) duration. After control records, the bath chambers were filled with various concentrations of local anesthetics and the action potentials recorded at 5-min intervals. The decrease of action potentials of the B- and C-fibers was recorded separately.

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G.1.1.10

Measurement of Sodium and Potassium Conductance in Voltage Clamp Experiments

PURPOSE AND RATIONALE

Voltage clamp experiments on single nodes of Ranvier can be performed with single myelinated nerve fibers from the sciatic nerve of the frog (Stämpfli 1954, 1968; Nonner 1969; Nonner et al. 1975; Stämpfli and Hille 1976; Borchard and Drouin 1978). Sodium, potassium and leakage currents (I_{Na} , I_K , I_L) can be measured.

Descriptions of structure and function of voltage-gated sodium channels were given by Catterall (1988, 1992), Stühmer (1991), Narahashi and Herman (1992), Cohen and Barchi (1993).

PROCEDURE

Single myelinated nerve fibers are dissected from the sciatic nerve of the frog (*Rana esculenta*). The fibers

are bathed in a Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 5.0 mM Tris, pH 7.3, at 15°C. For measurement of sodium, potassium and leakage currents (I_{Na} , I_K , I_L), the nodal membrane is clamped to a holding potential of -30 mV (relative to the resting potential of -70 mV). Hyperpolarizing prepulses (-40 mV, 50 ms) followed by 2 ms test pulses (60 mV) are applied every 5 s to record peak sodium currents, I_{Na} , and to avoid the influence of accumulation of frequency-dependent block. Every second pulse is followed by an afterpulse (120 mV, 20 ms) to measure steady state I_K . The leakage current, I_L , is measured at the end of the hyperpolarizing prepulse and recorded every 10 s.

After superfusion of the nodal membrane with a local anesthetic, a quick and then a slow decrease of the peak sodium current (I_{Na}) occurs. When the application of the local anesthetic is stopped, I_{Na} increases quickly and then slowly within min indicating a reversible action of the local anesthetic on the nerve fiber. With local anesthetics, the potassium current (I_K) behaves similarly, whereas the leakage current (I_L) remains unchanged.

Dose Dependent Action of Local Anesthetics on I_{Na} -V Relations

The holding potential is set at -30 mV. A hyperpolarizing prepulse (-40 mV, 50 ms) is followed by a depolarizing test pulse (30 ms) with varying amplitudes. Local anesthetics induce a dose-dependent reduction of sodium-inward and -outward currents over the whole range of voltage clamp steps. The measurements are performed at extracellular pH 7.3.

Dose Dependent Action of Local Anesthetics on I_K -V Relations

Potassium currents, I_K , are measured at the end of depolarizing test pulses of 30 ms duration. Local anesthetics induce a dose-dependent decrease of potassium currents at extracellular pH 7.3.

EVALUATION

The effects on sodium, potassium and leakage currents (I_{Na} , I_K , I_L), the dose dependence of action on I_{Na} -V relations, and the dose dependence of action on I_K -V relations of various local anesthetics are compared.

MODIFICATIONS OF THE METHOD

Bräu et al. (1998) determined half-maximal blocking concentrations of local anesthetics for tonic block of Na⁺ and K⁺ channels by using the axonal patch clamp

method (Vogel and Schwarz 1995) to study potencies of clinically used local anesthetics in suppressing Na⁺ and delayed rectifier K⁺ channels of peripheral nerve.

Sciatic nerves of the clawed toad *Xenopus laevis* were enzymatically prepared (Jonas et al. 1989). The nerves were dissected, desheathed, and treated with collagenase and protease to obtain single, partially demyelinated fibers. For recording axonal ionic currents, the outside-out configuration of the patch clamp method (Hamill et al. 1981) was applied to partially demyelinated axons. Patch pipettes were pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK) and coated with Sylgard 184 (Dow Corning, Seneffe, Belgium) and were fire-polished at the tip before use. The resistance of the pipettes after filling with internal solution was 15–25 MΩ. The bath solution contained (in mM): NaCl 115; KCl 2.5; CaCl₂ 2, HEPES 10; adjusted to pH 7.4 with TRIS. For Na⁺ current recording, 10 mM tetraethylammoniumchloride was added to suppress K⁺ currents; for K⁺ recording, 100 nM tetrodotoxin was added to suppress Na⁺ currents. The pipette solution for Na⁺ current recording contained (in mM): CsCl 110; NaCl 13; EGTA 2; HEPES 10, pH adjusted to 7.2 with TRIS. For the investigation of K⁺ channels, CsCl was replaced by KCl.

Different concentrations of local anesthetics were applied to the patches using a multi-barrel perfusion system that allowed solution changes within 1 s.

After forming an outside-out patch from the nodal membrane, the holding potential was adjusted to -90 mV in the voltage clamp mode using an EPC7 patch clamp amplifier (List, Darmstadt, Germany). The pulse protocol used to elicit Na⁺ currents comprised a 50-ms prepulse to -130 mV to remove fast inactivation, followed by a 50-ms test pulse to -40 mV to activate Na⁺ currents. Steady-state K⁺ currents were activated by a 50-ms potential step to 60 mV and were measured as the mean current during the last 20 ms of the pulse. For analysis, currents were filtered with a four-pole Bessel filter, digitized with a Labmaster TM-40 AD/DA board (Scientific Solutions, Solon, Ohio, USA), and stored in the hard disk of a computer. To reduce the noise of single current traces that originated from individual channel openings, 20 successive traces, elicited every 5 s, were averaged to measure the peak Na⁺ current or steady-state K⁺ current. Blocking potencies of the local anesthetics were determined by analyzing concentration-inhibition curves, which were constructed by measuring the inhibition of the peak Na⁺ current or steady-state K⁺ current amplitude at different concentrations normalized to control.

Half-maximal inhibiting concentrations (IC_{50}) were obtained from non-linear least-square fits of the function $F_1 = b_{\max}/(1+[IC_{50}/c]^p)$ to the data points. As first-order binding kinetics of the drug with the channel were assumed, the Hill coefficient (p) was set to 1 during fitting; b_{\max} is the maximal achievable block. Data acquisition and evaluation were performed with pClamp 5.5.1 (Axon Instruments, Burlingame, Calif., USA); Fig.P 5.0 software (Biosoft, Cambridge, UK) was used for fitting procedures.

Olschewski et al. (1998) applied the patch clamp technique to intact lateral horn neurons from laminae I–III identified in 200- μ m slices of spinal cord from newborn rats. Under voltage clamp conditions, the whole-cell Na^+ and K^+ currents activated by depolarization were recorded in the presence of different concentrations of anesthetics.

Olschewski et al. (2002) combined the patch clamp recordings in spinal cord slices of the rat with the “entire soma isolation” method, as described by Safranov et al. (1997) and Safranov (1999). In the whole-cell recording mode, the entire soma of the neuron was isolated from the slice by slow withdrawal of the recording pipette, leaving all or nearly all of its processes in the slice. The isolated structure was classified as *soma* if it had lost all its processes during isolation and preserved only 10%–20% of the original Na^+ current recorded from the neuron in the slice before its isolation. The isolated structure was considered as *soma* + *axon* complex if it contained one 10- to 100- μ m process and preserved >85% of the original Na^+ current recorded from the neuron in the slice before isolation. The structure was considered as *axon* + *dendrite* if it preserved one adjacent process but the amplitude of the Na^+ current was in the range of those typically seen in isolated *somata*. A successful isolation was usually accompanied by a considerable decrease in membrane leakage currents due probably to the loss of the large area of the dendritic membrane. The good physiological state of the isolated structures was confirmed by a considerable increase in their output resistances (reflecting a decrease in membrane leakage conductance), and by stable and even improved membrane resting potentials. Three types of tetrodotoxin-sensitive voltage-gated Na^+ channels on soma and processes of dorsal horn neurons of rat spinal cord were identified. Bupivacaine, lidocaine and mepivacaine at low concentrations (1–100 μ M) enhanced delayed-rectifier potassium current in intact neurons within the spinal cord slice, while exhibiting a partial blocking effect at higher concentrations (> 100 μ M).

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G.1.1.11

Inhibition of Fast Axonal Transport

PURPOSE AND RATIONALE

Local anesthetics block not only nerve conduction but also fast axonal transport (Aasheim et al. 1974; Lavoie 1983; Lavoie et al. 1989). This mechanism may also be the way in which local anesthetics cause injury to nerve and spinal cord (Kalichman 1993).

PROCEDURE

The biological material consists of the eighth and ninth dorsal root ganglia of the bullfrog (*Rana catesbeiana*) and their respective spinal nerves. Each side of the animal provides one such set of ganglia and associated structures. The dissection is performed at room temperature with the preparation in an oxygenated medium with a pH of 7.1 and of the following composition (in mM): NaCl, 114; KCl, 2.0; CaCl₂, 1.8; dextrose, 5.5; HEPES 20. Once the dissection is completed, each spinal nerve is ligated at approximately 24 mm from the ganglion.

The ganglia are pulse-labeled with [³H]leucine for 1 h. After the pulse-labeling, the preparations are incubated for 16–17 h at 18°C in medium free of radioactivity; the two ganglia and spinal nerves from one side of the animal serve as control preparations and those from the other side as experimental preparations. The preparations are placed in a Lucite incubation chamber. The ganglion and the first 10 mm of the nerve trunk occupy one compartment, the next 3 mm is in the groove between two communicating compartments, and the rest of the nerve trunk (including the point of ligation) is placed in the second compartment. The two communicating compartments are then sealed

from one another by filling the rest of each groove with silicone grease. The compartment containing the control ganglia and that containing the experimental ganglia are filled with medium for all experiments. A local anesthetic agent is added to the medium bathing the distal half of the experimental nerve trunks whereas the same area of the control nerve trunks is in the corresponding medium without local anesthetic.

At the end of the incubation, the nerves are cut into 2-mm segments, and the protein-bound radioactivity of each ganglion and nerve segment is determined by liquid scintillation counting (Lavoie 1981). For each preparation, the total quantity of transported radioactivity is calculated by summing up the radioactivity present in the individual segments of the nerve starting at 6 mm from the ganglion and extending up to the point of ligation. The total quantity of [³H]leucine incorporated into protein is calculated for each preparation as the sum of transported radioactivity and radioactivity remaining in the ganglion.

EVALUATION

Mean values ± SEM for the ratio of radioactivity in the experimental preparation to radioactivity in the control preparation are computed in each pair of preparations for the total incorporated radioactivity, the total transported radioactivity, and the radioactivity at the ligation. The difference in the absolute amount of radioactivity at the ligation, the difference in total transported radioactivity, and the difference in total incorporated radioactivity are calculated for each pair of preparations. A Student's *t*-test for paired samples is performed on the mean differences between control and experimental preparations. Using the individual ratios of radioactivity in experimental preparation to control preparation, an analysis of variance on parallel groups is done to compare the drugs; pairwise comparisons are obtained using Scheffé's critical *F* value.

MODIFICATIONS OF THE METHOD

Anderson and Edström (1973) and Lavoie (1982) used frog sciatic nerves *in vitro* to study the effect of local anesthetics on fast axonal transport of proteins. Rabbit vagus nerves were used by Fink et al. (1972) and Aasheim et al. (1974).

Factors, such as kinesin and dynein, influencing fast axonal transport have been identified (Shea and Flanagan 2001).

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G.1.2

Infiltration Anesthesia

G.1.2.1

Infiltration Anesthesia in Guinea Pig's Wheals

PURPOSE AND RATIONALE

Based on earlier work by McIntyre and Sievers (1937) and Sievers and McIntyre AR (1938), the use of intracutaneous wheals in guinea pigs was recommended by Bülbring and Wajda (1945). This method has become a standard operating procedure for testing local anesthetics.

PROCEDURE

Adult guinea pigs of either sex weighing 250–300 g are chosen. On the day preceding the experiment the hair on the back is clipped and two areas of 4–5 cm diameter are shaved. This produces a certain amount of irritation which disappears overnight. The sensitivity of the skin is greatest in the midline and slightly more so in the front than in the back area. For this reason each concentration of a local anesthetic must be tested in both areas. Six tests using three guinea pigs can be performed simultaneously. The doses of local anesthetics are always injected intracutaneously in 0.1 ml saline. Three guinea pigs receive one dose in the front area and another dose in the back area; the size of the wheal is marked with ink. One side is used for the test preparation, the other side for the standard (e. g., 1% butanilcaine). The reaction to pin prick is tested 5 min

after injection in the following way. After observing the animal's normal reaction to a prick applied outside the wheal, six pricks are applied inside the wheal and the number of pricks is counted to which the guinea pig fails to react. The pricks are applied at intervals of about 3–5 s. Six pricks are applied every 5 min for 30 min. Having completed the test on 3 guinea pigs, the same solutions are injected into 3 other guinea pigs, but the solution which was used for the front is now used for the back area and vice versa.

EVALUATION

The number of times the prick fails to elicit a response during the 30 min period is added up, and the sum, out of possible 36, gives an indication of the degree of anesthesia. Using various doses, dose-response curves can be established. For time-response curves, the prick tests are repeated every 10 min. Half-life times are calculated as the time, when after complete anesthesia 3 out of 6 pricks elicit again a response (Ther 1953a, b).

MODIFICATIONS OF THE METHOD

The test can also be used to study the influence of vasoconstrictors, such as adrenaline.

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G.1.2.2**Infiltration Anesthesia in Mice****PURPOSE AND RATIONALE**

Grant et al. (2000) described a mouse model for evaluation of skin anesthesia after infiltration of local anesthetics.

PROCEDURE

Male Swiss Webster mice weighing 23 ± 3 g are used. On the day preceding the experiment, the abdominal hair is shaved using electric clippers. On the day of the experiment, on either side of the abdomen (~ 0.5 cm lateral to the midline and ~ 1.0 cm below the thoracic cage) circular areas of ~ 0.7 cm diameter are marked with indelible ink for subcutaneous injections. For the generation of nociceptive electrical stimuli, a square wave general purpose generator (model S48, Grass Instruments) coupled to an isolator unit (Model PSIU6 F, Grass Instruments) is used. The stimuli are delivered to the skin via blunt electrodes fashioned from 25-gauge needles. A thin layer of electrically conductive gel is applied to the area to be tested with a cotton-tipped applicator.

Mice are screened to determine their vocalization threshold, the current required to produce a vocalization (squeak). While nociceptive stimulus is applied, animals are restrained gently in the experimenter's palm by grasping the nape of their neck and their tail. The output of the stimulator is initially set at 5 mA, and is progressively increased in steps of 1 mA. Mice are screened once, and those animals that vocalized at ≤ 8 mA are included in the study.

The local anesthetics are injected in various concentration subcutaneously in a volume of 0.15 ml in the marked area of one side. After fixed time intervals (5, 10, 20, 30, 40, 50, 60, and 70 min) the skin overlying the injection site and the corresponding "control" side are stimulated using the threshold stimulus. If the animal does not vocalize after the threshold stimulus was applied to the test site, the intensity of the stimulus is increased twice in 1-mA increments, to deliver a "maximal" stimulus 2 mA above the threshold. This is done to ensure lack of vocalization response.

To assess the duration analgesia is tested at 15-min intervals.

EVALUATION

The all-or-none response data are subjected to statistical analysis using the survival curves procedure. The curves are generated using the method of Kaplan and Meier, and the 95% confidence interval for fractional

survival at any particular time is calculated using the Prism program (GraphPad Software). The curves are compared using the log rank test. In this method of statistical analysis, the term "survival" refers to any well-defined end-point and in the present study it is lack of analgesia. The two-tailed p value of < 0.05 is considered significant.

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G.1.3**Surface Anesthesia****G.1.3.1****Surface Anesthesia on the Cornea of Rabbits****PURPOSE AND RATIONALE**

Following the pioneering work of Sollmann (1918), block of the rabbit corneal reflex as described by Régnier (1923) has become a standard test method for evaluating local anesthetics (Quevauviller 1971).

PROCEDURE

Albino rabbits of either sex weighing 2.5–3 kg are placed into rabbit holding cages. The upper and lower eyelashes are carefully clipped. The conjunctival sac of one eye is held open, thus forming a pocket. From a 1 ml syringe with a 22-gauge needle, 0.5 ml of a solution of the anesthetic is applied into the conjunctival sac for 30 s. Then the procedure is repeated, so that 1.0 ml is applied within 1 min. One ml of the standard (0.1% solution of tetracaine hydrochloride) is applied to the other eye. Effective local anesthetics extinguish the corneal reflex (blinking) elicited by any touch of the cornea. For quantitative purposes, the irritation with a bristle according to von Frey (1894, 1896, 1922) has been recommended. An equine hair bending at a load of 230 mg is attached perpendicularly to a glass rod. Within 25 s, the cornea is touched 100 times. The summation of many stimuli applied this way gives better results than a single touch with a glass rod (Ther and Mügge 1953). The test is started 5 min after application of the drug and repeated every 5 min until anesthesia vanishes and blinking occurs again. The time between disappearance and reappearance of the corneal reflex is registered.

EVALUATION

Using the time of loss of the corneal reflex as parameter after application of different doses, dose-response curves can be established and potency ratios versus the standard calculated.

MODIFICATIONS OF THE METHOD

Chance and Lobstein (1944) used **guinea pigs** for testing surface anesthesia by the corneal reflex.

Bartsch and Knopf (1970) described an electrically operated stimulator with a bristle allowing variable frequencies to evaluate surface anesthesia on rabbit cornea.

Hotovy (1956) investigated the synergism between the local anesthetic compound dibucaine hydrochloride applied to the conjunctival sac of rabbits and intravenously administered analgesics using Régnier's method

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G.1.3.2

Suppression of Sneezing Reflex in Rabbits

PURPOSE AND RATIONALE

Nieschultz et al. (1958), Åström und Persson (1960) used the sneezing reflex in rabbits to test local anesthetic activity.

PROCEDURE

Groups of male rabbits weighing 3 kg are used. Using a cotton tampon, the test solution is applied to the mucous membrane of one nostril. The solution of a standard local anesthetic is administered to the nasal mucosa of the other nostril. After 2 min the mucous membrane is stimulated by a fine pencil. Loss of the sneezing reflex is regarded as sign of complete anesthesia. The stimulation is repeated after 3, 6, 10 and 15 min and continued every 5 min until the sneezing reflex reappears. Various concentrations of test compound and standard are applied.

EVALUATION

Using the loss of the sneezing reflex as parameter after application of different doses, dose-response curves can be established and potency ratios versus the standard calculated. Furthermore, the duration of activity can be evaluated.

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G.1.4**Epidural Anesthesia****G.1.4.1****Epidural Anesthesia in Various Species****PURPOSE AND RATIONALE**

Activity and tolerability of new local anesthetics after epidural injection have to be studied in various animal species in order to predict both parameters in patients.

PROCEDURE***Studies in Rats***

Blomberg and Rickstein (1988) described a simple technique for cannulation of the thoracic epidural space in conscious and anesthetized rats. Male Wistar rats weighing 300–400 g were anesthetized with methohexital sodium 100 mg/kg intraperitoneally for insertion of PE-50 catheters into the tail artery and right jugular vein. Supplementary doses of methohexital were given during the cannulation of the epidural space. The catheters were exteriorized in the neck and the animals were placed in a lucite restraining cylinder where they were left to recover for 1–2 h from methohexital anesthesia and cannulation procedures. Arterial pressure, heart rate, and body temperature were recorded. A PE-50 catheter was inserted into the right common carotid artery for measurement of cardiac output with the cardiogreen dye-dilution technique and measurement of left ventricular end-diastolic pressure.

For cannulation of the thoracic epidural space, the skin of the upper part of the back of the animal was shaved and a 3-mm-long midline incision was made over the spinous process from a point between the two scapulae. The fascia covering the superficial muscles of the back was opened and the muscles were dissected from the thoracic vertebrae and retracted laterally. The thick and protruding spinous process of the second thoracic vertebra was localized. The identification of the spinous process of Th3 to Th7 was easily made by the nail of the index finger. The interspinal muscles and ligaments between the spinous processes of Th6 to Th7 were cut and the spinous process of Th7 was removed. The distance between Th6 and Th7 was then maximized by a rostral traction of Th6 with a forceps attached to spinous process of Th6. The yellow ligament between Th6 and Th7 was identified by a dissecting microscope at $\times 16$ magnification and cut open to visualize the dura and the spinal cord. A PE-10 catheter, filled with the local anesthetic, was introduced cranially to a length of 1 cm. The area between Th6 and Th7 was covered and the catheter was firmly attached

with cyanoacrylate, which completely prevented extradural leakage of the local anesthetic. The catheter tip was now located at the Th4–5 level. The superficial muscles were then sutured and the catheter was brought through the closed skin incisions. Incremental doses of 10 μ l of bupivacaine were administered epidurally and the amount of the anesthetic bupivacaine (5 mg/ml) that caused no further heart rate decrease was assessed. The spread of the analgesia was assessed by a modified pin-prick technique. All hemodynamic measurements were made before, and then 10–15 min and 45–55 min after the induction of thoracic epidural anesthesia.

Studies in Guinea Pigs

A method to test epidural anesthesia in guinea pigs was described by Åkerman et al. (1988).

Male guinea pigs weighing 300–500 g are anesthetized by means of an intraperitoneal injection of an aqueous solution of chloral hydrate 42.5 g/l; ethanol 90 g/l; propylene glycol 428 g/l; sodium pentobarbitone 9.75 g/l; and magnesium chloride 21 g/l. A skin incision is made from the level of the lumbosacral fossa and approximately 1.5 cm down in order to expose the sacral area in the mid-line. With the vertebral column flexed, the lumbosacral intervertebral ligament is carefully incised. Through this small opening a polyethylene catheter (PE 10) is inserted maximally 1.5 cm along the roof of the vertebral canal to the L4–L5 region. The catheter is sutured to the overlying lumbar fascia which is then closed. The catheter is tunneled under the skin and exteriorized through an incision in the neck region. After fixation of the catheter to the fascia of the neck muscles and suturations of the incisions, the catheter is filled with saline and sealed.

After a recovery period of at least 1 day, 0.1 ml of 2.0% lidocaine is injected over a period of 1 min, and the motor and sensory blocks are assessed. The injection of lidocaine which results in a bilateral, reversible blockade indicates a successful preparation. A minimum of 8 animals are used in the further experiments for each test solution.

Mean time to onset of block and mean duration of block are calculated from number of legs blocked.

Siems and Soehring (1952) described a model in **guinea pigs** resembling peridural and paravertebral anesthesia in man.

Studies in Rabbits

Chernyakova et al. (1994) studied the effects of azacaine during epidural anesthesia in **rabbits**.

Hughes et al. (1993) described a rabbit model for the evaluation of epidurally administered local anesthetics. A "loss of resistance technique" similar to that employed in caudal epidural injection in humans was used. The rabbit was carefully restrained by an assistant. The readily palpable cranial dorso-iliac spines, lying on either side of the prominent spinous process of the seventh lumbar vertebra, served as landmarks.

The thumb and the middle finger of the left hand were placed on the two crests and the left index finger used to palpate the midline, L7 spine and the depression over the lumbosacral fossa. With the index finger in position on the L7 spine to serve as a guide, a short bevel 1.5 cm 20 gauge spinal needle was introduced at a right angle to the skin in the midline with the bevel aligned longitudinally. After passage through the skin, only minor resistance was felt until the ligamentum flavum was reached. When passing through the ligament, a definite "pop" was felt and resistance to advancement of the needle was lost. When correctly placed, the needle was at a depth of approximately 0.75 to 1.0 cm and firmly held by the ligament. The stylet was then withdrawn and the hub inspected for the presence of blood or cerebrospinal fluid. If absent, the needle was rotated through 90° to direct the bevel caudally, a 1.0 ml syringe was attached and 0.1 ml of air injected. Accurate placement was indicated by the absence of resistance to injection and lack of subcutaneous crepitus. A syringe containing the desired dose of the local anesthetic was attached and the solution injected over a 5–10 s period. The pharmacodynamic responses were assessed by (1) sensory loss, (2) loss of weight-bearing ability, and (3) flaccid paresis.

Studies in Dogs

Kief and Bähr (1970) studied the epidural tolerance of artecaine with and without addition of Suprarenin in Beagle dogs weighing 9–12 kg. For pre-operative sedation, the dogs received 0.03 ml/kg Combelen (= propionylpromazine) intravenously. The fur of the lumbosacral area was shaved and the skin disinfected. A single dose of 5 ml of a 2% artecaine solution was administered epidurally under sterile conditions. All dogs showed the typical symptoms of spinal anesthesia, which subsided after a few hours. The dogs were sacrificed after 1 or 3 days. The portion of the vertebral column with the site of injection in the middle was removed and placed in 8% buffered formalin. When semifixed, the vertebral arches were opened and the spinal cord as well as the roots of the spinal nerves with the adipose tissue of the epidural space were re-

moved. After embedding in gelatin and Paraplast, the serial sections from the area of injection were stained with fast red 7B, hematoxylin-eosin and myelin sheath staining after Olivecrona was performed. Furthermore, the PAS and iron reaction were performed on one section. The presence or absence of nerve damage and of inflammation was noted.

A technique for epidural administration in the **dog** was described by Feldman and Covino (1988).

Defalque and Stoelting (1966) used a standard veterinary epidural technique to study latency and duration of action of some local anesthetic mixtures in **dogs**. The animals, prone and with their spread-out extremities attached to the table, were placed in 40-degree Trendelenburg position. Under sterile conditions, the epidural space was penetrated through the interarcual ligament with a short-beveled No. 22 spinal needle. After identification of the epidural space (aspiration, air injection), 4 ml of the investigated solution were injected with a constant injection rate of 2 ml per sec. Absence to skin-twitch response to pinching with an Allis clamp was considered analgesia. This was tested on both flanks, along a line 2 cm off the spine. Disappearance of contraction of the anal sphincter in response to stroking all quadrants of the anal margin closely correspond to complete analgesia at the level of the interarcual ligament; since this was an easily measurable parameter, disappearance and recurrence of this reflex was chosen as endpoint of latency and duration of action, respectively.

Raner et al. (1994) performed thoracic epidural anesthesia in **dogs** after general anesthesia with thiopental/chloralose and under artificial respiration. A thoracic epidural catheter (18G, Braun-Melsungen, Germany) was inserted percutaneously via the Th7–8 or Th8–9 interspace. Thoracic epidural anesthesia was established with a bolus injection of 10 mg/kg mepivacaine followed by a continuous infusion of 5 mg/kg per hour.

Kamibayachi et al. (1995) studied the halothane-induced myocardial sensitization to the dysrhythmogenic effect of epinephrine after thoracic epidural anesthesia in **dogs**. An epidural catheter was inserted under halothane anesthesia. The vertebral arches of T8 and T9 were surgically exposed and the catheter was introduced, via the T8–9 interspace, to the epidural space, which was identified by the loss-of-resistance technique. The catheter was advanced 5 cm in the cephalad direction and secured to the back. After the experiment, the position of the catheter was confirmed radiographically by injection of iopamidol (0.2 ml/kg).

Studies in Other Species

Ide et al. (2001) studied the effect of epidural anesthesia on respiratory distress induced by airway occlusion in isoflurane-anesthetized **cats**. The authors developed an animal model for the study of airway occlusion and proposed new concepts of minimum alveolar anesthetic concentration for airway occlusion, and the duration from the start of airway occlusion to the onset of the positive motor response. Adult cats were anesthetized with isoflurane and an epidural catheter was placed after L5 laminectomy.

Richer et al. (1998) described sacrococcygeal and trans-sacral epidural anesthesia in the laboratory **pig**. The aim of the study was to assess epidural accessibility in 30- to 50-kg piglets, not in adult hogs. Each animal received general anesthesia combined with epidural anesthesia. The level of catheter insertion was the sacrococcygeal interspinous space or the adjacent space (S4-S5). Localization of the injection site was simple. The sacrococcygeal junction corresponds to the junction between the fixed portion (sacrum) and the mobile portion (coccyx) of the animal's tail. The catheter was inserted along the median line at this level or the level above. The bony structures were palpable beneath the superficial planes. The animals were placed on their sides under general anesthesia and disinfected under surgical conditions. Epidural space injection was performed using a 16-gauge needle, traversing the sacrococcygeal or trans-sacral interspinous space. Entry to the epidural space was detected by a loss of resistance to puncture with absence of blood or cerebrospinal fluid on aspiration and by the ease of cephalic catheter progression within this space. A first injection of local anesthetic was performed 10 min prior to laparotomy. Epidural analgesia was maintained after this first injection by continuous administration of the same agent with a syringe. It was discontinued on awakening for a brief period in order to confirm functional recovery of the lower limbs.

Lebeaux (1975) recommended the **sheep** as a model for testing spinal and epidural anesthetic agents. To ensure accurate and reproducible administration of drugs into these restricted anatomical regions, a wooden stock was built to restrain the sheep without stress but allow arching of the lumbosacral spine in order to open the inter vertebral space. The stock consisted of a table with holes for the front feet, hinged at one end to a fixed stanchion. The hinges permitted tilt of the table. The sheep was placed prone on the table, the head secured in the stanchion and the front feet passed through the holes and tied. The lumbosacral interspace used for

both spinal and epidural tap was separated by positioning and tying the rear limbs cephalad beside the abdomen, thereby extending the knee and flexing the hip. The wool was clipped from the sheep's back, the skin of the lumbosacral area was washed and swabbed with iodine, and the vertebral spines and interspaces identified by palpation, marked, and numbered with a felt pen.

Epidural block was performed with a 19-gauge Crawford-point needle, using the loss-of-resistance method, with a syringe containing saline to identify the epidural space. After withdrawal of the stylet, a Luer-Lok syringe containing 10 ml of sterile saline solution with a final concentration of 1:200,000 epinephrine and 25.0 mg (0.25%) bupivacaine was attached to the needle. After careful aspiration of blood or spinal fluid, the local anesthetic solution was injected within 30 s. The needle was withdrawn and the table kept horizontal.

Immediately after injection, the rear feet were untied and allowed to hang free on either side of the table for evaluation of sensation and/or reflexes in the rear limbs. Then, 20 min after injection the sheep was removed from the table and positioned in a sling fashioned from a heavy duty 50-gallon plastic barrel split in half longitudinally and mounted on a metal support. Holes cut in the barrel allowed the feet to drop through so that comfortable sternal recumbency was maintained while permitting anesthetic evaluation to continue. When complete sensation returned in the skin of the dorsum, the sheep was removed from the sling and placed on a straw-bedded floor so that weight support and full recovery could be assessed.

Feldman et al. (1997) compared anesthetic efficacy and pharmacokinetics of epidurally administered ropivacaine and bupivacaine in the **sheep**.

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G.1.5

Intrathecal (Spinal) Anesthesia

G.1.5.1

Spinal Anesthesia in Various Species

PURPOSE AND RATIONALE

Transient neurological symptoms have been observed in patients after spinal anesthesia (Hampl et al. 1995). Myers and Sommer (1993) published a survey on methodology for spinal neurotoxicity studies.

Activity and tolerability of new local anesthetics after intrathecal injection were studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients.

PROCEDURE

Studies in Rats

Intrathecal injections to **rats** are performed according to the method of Hylden and Wilcox (1980) or Åkerman (1985) as being used by Ossipov et al. (1988). Male Sprague-Dawley rats weighing 50–75 g are used. The rat is held firmly by the pelvic girdle. A 30-gauge needle is attached to a 25- μ l Hamilton syringe is inserted into the tissue on one side of the L5 or

L6 spinous process at an angle of about 20°. The needle is advanced to the groove between the spinous and transverse processes and then moved forward the intervertebral space at an angle of about 10°. About 0.5 cm of the needle is then in the vertebral column. Correct placement of the needle is indicated by an arching of the tail. Drugs are dissolved in saline or water and administered in a volume of 5 μ l. Antinociception is determined in a modification of tail flick assay in rats by placing the tail of the rat under a focused radiant heat source. The degree of antinociception is defined as the percentage of maximum possible effect. This percentage is determined for each dose at each time measured allowing to calculate ED_{50} values.

Omote et al. (1995) studied the effects of verapamil on spinal anesthesia induced by local anesthetics administered via a chronic intrathecal polyethylene catheter in **rats**. The catheter was inserted 15 mm cephalad into the lumbar subarachnoid space at the L4–5 vertebrae with the tip located near the lumbar enlargement of the spinal cord. The catheter was tunneled subcutaneously and externalized through the skin in the neck region. At least 6 days of postsurgical recovery was allowed before animals were used in experiments. The tail-flick and the mechanical paw pressure tests were used to assess thermal and mechanical nociceptive thresholds.

Mestre et al. (1994) described a method for performing direct intrathecal injections in **rats** without introducing a spinal catheter.

Wang et al. (1991) described lumbar subarachnoid catheterization in rats. Male Wistar rats weighing 500–600 g were anesthetized with intraperitoneal ketamine 75–100 mg/kg. Following a midline skin incision, the paravertebral muscles were detached from the spinous processes and retracted laterally. The intervertebral ligament between Th13 and L1 or between L1 and L2 was removed. After dissection, the inferior border of T13 or L1 was retracted cephalad, facilitating the application of rongeurs for partial laminectomy at the cephalic border of L1 or L2. This exposed the dura and the underlying spinal cord, which was easily identified by a mid-line blood vessel. Lidocaine (1.5%) was applied topically to the dura to prevent movement of the animal when the dura was picked up with fine forceps. The dura was perforated with a short-bevel No. 20-gauge needle, resulting in some leakage of cerebrospinal fluid. Under magnification, a PE10 catheter was immediately inserted tangentially through the dural opening. It was directed caudally and maintained dorsal to the spinal cord. The catheter, 10 cm in length and containing a volume of 0.02 ml was advanced

slowly in the subarachnoid space to a mark 1.5 cm from the tip, while 0.01–0.02 ml of normal saline was simultaneously injected to open the way. Leakage of fluid through the dural opening during injection confirmed the patency of the catheter and also indicated that the catheter was properly placed in the subarachnoid space.

For fixation of the catheter, the spinous process rostral to the laminectomy was denuded and perforated with a No. 18-gauge needle. The free end of the catheter was threaded through this lumen. On retraction of the needle, the PE10 catheter was left in the perforation of the spinous process, and anchored with cyanoacrylate glue. A connector was attached to the free end of the catheter for injection.

The wound was irrigated with saline and closed in layers leaving the catheter buried in the subcutaneous tissue. The hub of the connector was sutured to the skin with fine stainless steel wire. The injection port, covered by a removable metal cap, was brought out of the skin via a separate small opening lateral to the main skin incision.

One week after surgery, 0.03–0.05 ml of 1.5% lidocaine was injected through the subarachnoid catheter. Correct positioning of the catheter was evidenced by prompt sensory and motor block of the hind limbs, developing in 1–5 min, and exhibiting motor blockade for 20–30 min.

Dirksen et al. (1992) studied the dose–response and time–effect relationships of intrathecal bupivacaine in rats. The effect was a quantified drug-induced and graded reduction in the magnitude of the withdrawal reflexes elicited by transcutaneous stimulation.

Sakura et al. (1996) described an improved technique for morphological analysis of drug-induced injury after intrathecal catheterization in the rat. Male Sprague Dawley rats weighing 200–300 g were anesthetized by intraperitoneal injection of methohexital (40–60 mg/kg). Catheters were composed of 28-gauge polyurethane, 32-gauge polyimide, 32-gauge polyurethane, PE10 polyethylene, or PE-10 polyethylene that has been stretched to twice its original length. They were passed through a slit in the atlanto-occipital membrane and advanced 11 cm, to lie with the tip caudal to the conus medullaris. Animals were allowed to recover for 24 h before the study was started. The animals were placed in a horizontal acrylic restraint, and baseline tail-flick latency was assessed immediately before infusion. Infusions were administered via a mechanical infusion pump at a rate of 1 μ l/min for 4 h. Animals were evaluated for alteration in tail-flick latency 7 days after infusion.

Animals were sacrificed by injecting an overdose of pentobarbital 7 days after infusion. They were perfused intracardially with a phosphate-buffered glutaraldehyde-paraformaldehyde fixative. The spinal cord and nerve roots were dissected out, immersed in the same glutaraldehyde solution for infusion fixation and embedded in glycol methacrylate. The embedded tissue was sectioned at the conus medullaris, 6 mm rostral, and 6 and 12 mm caudal, using a Sorvall JB-4 microtome. The tissue was stained with hematoxylin-eosin or was treated with 4% osmium tetroxide and stained with toluidine blue. Histologic evaluation was performed with light microscopy. Specimens obtained 12 mm caudal to the conus were used for quantitative comparison of catheter-induced damage. This region was chosen because cross sections obtained below the conus have a greater number of fascicles. Each fascicle present in the cross-section was assigned to injury scores of 1 to 3, where 0 = normal, 1 = mild, 2 = moderate, and 3 = severe injury. The injury score of each cross-section was then calculated as the average score of all fascicles present in the cross-section. The data were compared using the Kruskal-Wallis test and Dunnett's test. For all comparisons, $P < 0.05$ was considered significant.

Grouls et al. (1997) compared the effects of *n*-butyl-*p*-aminobenzoate and bupivacaine after epidural and intrathecal solution in the rat. Anesthesia was measured by the latency of tail withdrawal from warm water (55°C).

Kirihara et al. (2003) compared the **neurotoxicity** of intrathecal and epidural lidocaine in rats. Male Sprague Dawley rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and 1.5% halothane. A catheter of stretched polyethylene tubing PE10 was introduced into the subarachnoid or epidural space using an aseptic technique. Catheters were passed via the L4–L5 intervertebral space and advanced 1.3 cm in the caudal direction. Rats were allowed 4 days to rest for recovery from the operation.

To measure the response to noxious heat stimulus, a tail-flick test was performed. A 100-W projector lamp was focused on the distal segment of the tail approximately 5 cm from the tip. The time at which rats withdrew the tail was defined as the tail-flick latency. A cutoff time of 10 s was used to avoid damaging the tail.

To measure the response of legs to a noxious mechanical stimulus, a paw pressure test was applied to the dorsal surface of both hind paws using a device capable of progressively increasing the pressure at a rate of 15 g/s. The pressure at which the rat withdrew the

paw from the device was defined as the paw pressure threshold, and the mean for both paws was used for analysis. A cutoff pressure of 400 g was used to prevent damage to the paws.

Motor function in the lower limbs was assessed by grading: 0 = none, 1 = partially blocked, and 2 = completely blocked.

Various concentrations of local anesthetic or saline were injected intrathecally in a volume of 20 μ l or epidurally in a volume of 100 μ l followed by 10 μ l saline to flush the catheter. Tail-flick test, paw pressure test and motor function assessment were performed 10, 20, 30, 60, 120, 180, and 240 min after injection and continued daily for 4 days.

After the last experiments, the rats were euthanized by injection of an overdose of pentobarbital and then perfused intracardially with a phosphate-buffered 2.0% paraformaldehyde/2.5% glutaraldehyde fixative. Methyl green solution was injected to confirm the location of the catheter after the perfusion. The spinal cord and nerve roots were dissected out and immersed in the same fixative for 4 h. Two specimens (10 mm rostral and caudal to the conus medullaris from each rat) were postfixed with cacodylate-buffered 1% osmium tetroxide, dehydrated in a series of graded alcohol solutions, and embedded in epoxy resin. From the embedded tissue, 1- μ m transverse sections were obtained and stained with toluidine blue dyes. Sections obtained from 10 mm rostral to the conus (caudal spinal cord) were used for qualitative evaluation. Quantitative analysis of nerve injury was performed using the sections obtained from 10 mm caudal to the conus. Each fascicle present in the cross-section was assigned an injury score of 0 to 3. The injury score for each cross-section was then calculated as the average score of all fascicles present in the cross-section.

Data are presented as mean \pm SEM. Tail-flick latencies and paw pressure thresholds were converted to the percentage of the maximal possible effect. The area under the time-effect curve was calculated by accumulating the effect measured at discrete time intervals using the trapezoidal integration method. The results were analyzed by ANOVA with repeated measures followed by Scheffé and Dunnett tests. The injury score for each technique and each solution was compared using two-way ANOVA followed by the Scheffé test. The frequency (i.e., the number of rats with lesions) in each group was analyzed by the chi-square test.

De la Calle and Paíno (2002) described a procedure for direct **lumbar puncture in rats**. Female Sprague Dawley rats weighing 180–250 g were used. The rats

were placed in a transparent plastic box and anesthetized with a mixture of 4% isoflurane in O₂:N₂O (30:70 v:v). Then, a mask was placed over the rat's nose and mouth, and the isoflurane concentration was lowered to 1.5%–2% for the remainder of the procedure. The lower half of the animal's back was shaved and scrubbed with povidone-iodine. The animals were then placed in a prone position on a Styrofoam board (25 cm \times 13 cm \times 6 cm), with the mask opening fixed 12 cm from the end of the board. The rat's forelimbs were extended to the front and fixed to the board with tape, taking care not to force the neck. The hind limbs were left to hang off the board, lying on the table. In that way, the animal's vertebral column was flexed around the L3–L5 level, widening these intervertebral spaces. Using the anterior part of the iliac crest as a tactile landmark for the L5–L6 intervertebral level, a 2-cm longitudinal incision was made with a scalpel rostral to this point. The fascia of the paravertebral muscles was excised and withdrawn to the sides with the help of sterile cotton plugs. A neonatal lumbar puncture needle (25 G \times 1 TW) was introduced perpendicular to the surface through the widest intervertebral space and lowered until it came into contact with the vertebral body. Occasionally, a short flicking of the tail or of a limb was observed. The moment of penetration into the intrathecal space could be detected by a change of resistance to the introduction of the needle. Normally, the bevel of the needle was oriented rostrally although, if the puncture was not initially successful, the needle was tentatively rotated. A few seconds after withdrawal of the inner needle filament, the cerebrospinal fluid (CSF) could be observed to spontaneously flow into the needle cup. Triggering or accelerating liquid flow could be achieved by: (1) pressing the neck of the animal with a finger (thereby increasing the intrathecal pressure), (2) holding the head of the rat secure with one hand and pulling its tail with the other (the mechanical traction accommodates the needle tip in the intrathecal space), and (3) changing the inclination of the board to about 45° (also increases intrathecal pressure).

The procedure can also be used for the delivery of chemical products or cell suspensions to the intrathecal space. After collecting 20–50 μ l CSF, 50 μ l of 2% mepivacaine was injected. Hind limb paresis and anesthesia were produced that lasted for 30 min.

Bahar et al. (1984a) described chronic cannulation of the intradural and extradural space in the **rat**. Under anesthesia a hole was drilled in the penultimate lumbar vertebra of male rats and the appropriate space can-

nulated. The catheter was tunneled subcutaneously to emerge at the neck.

Using this technique, Chanimov et al. (1997) studied neurotoxicity after spinal anesthesia induced by serial intrathecal injections of magnesium sulfate. Male Wistar rats were given intrathecal injections of 0.02 ml of 6.3% or 12.6% magnesium sulfate or 2% lignocaine or 0.9% sodium chloride as a series of 15 injections on alternate days for a period of 1 month.

Yaksh and Rudy (1976) described a procedure of chronic catheterization of the spinal subarachnoid space in **rats** and **rabbits**. A polyethylene catheter (PE-10) was inserted through a puncture of the atlanto-occipital membrane into the spinal channel in anesthesia and secured to the skull. In this way, drugs could be administered into the spinal subarachnoid space of unanesthetized animals.

Cole et al. (1990) used this model to determine the influence of spinal tetracaine on central nervous system metabolism during nociceptive stimulation in **rats**.

Studies in Mice

Åkerman et al. (1988a, b) used **mice** to study spinal morphine antinociception potentiation by local anesthetics.

A simple technique for intrathecal injections by lumbar puncture in unanesthetized **mice** was described by Hylden and Wilcox (1980).

This model has been used by Langerman et al. (1994) to evaluate the potency of various local anesthetics. Adult Swiss Webster male mice were slightly anesthetized with halothane and the skin overlying the dorsal lumbar spine was opened using a transverse incision 8–10 mm. Animals were allowed to recover for 1 h before the evaluation of the baseline nociceptive response latency. Thirty min after baseline testing, intrathecal local injections were performed with an automatic syringe fitted with a 30-gauge needle. Fourth spinal space was identified and the needle was inserted between the two spinal processes in a cephalad direction at a 20° angle. Resistance encountered at approximately 5–6 mm from the skin indicated proper location of the needle in the spinal canal. A fixed 10 µl volume of injectate was used. Analgesia was measured using the tail-flick test based on tail withdrawal in response to heat generated by a light beam focused on the ventral tail surface.

Using this model, Åkerman (1985) obtained dose–response curves for lignocaine, mepivacaine, bupivacaine, amethocaine and cinchocaine after a single intrathecal injection in **mice**.

Studies in Rabbits

Bieter et al. (1936a) described a method of inducing spinal anesthesia in the **rabbit** and determined threshold anesthetic and lethal concentrations. Injections were performed at the lumbo-sacral union between the spinous process of the last lumbar vertebra and the first sacral spinous process. A dose of 0.02 ml per centimeter of spinal length was chosen as standard volume for determining minimal anesthetic and minimal lethal doses. These authors (Bieter et al. 1936b) determined the duration of spinal anesthesia in the rabbit after applying an electrical stimulus to the skin of the animal and observing the changes in respiration.

This method was also used by Luduena et al. (1960).

Langerman et al. (1991) studied the duration of anesthesia after a single subarachnoid injection of a local anesthetic in **rabbits**. Tetracaine 1% 0.5 mg/kg was administered in 10% glucose or in lipid solution via catheters chronically implanted in the subarachnoid space. The pharmacologic effect was assessed by evaluating the intensity and duration of motor blockade according to a three-stage scale.

Wakamatsu et al. (1999), Ohtake et al. (2000), and Oka et al. (2001) studied the effects of intrathecally administered local anesthetics on glutamate release and neuronal injury in **rabbits**. New Zealand white rabbits were anesthetized with isoflurane. With the rabbits in prone position, midline skin and subcutaneous fascia were incised between the third lumbar and the first sacral spinous process after infiltration with 0.25% bupivacaine. Muscles were dissected; the third to seventh processes, ligamentum flavum and epidural fat were sequentially removed; and the underlying dura was exposed. Using an operating microscope, a small slit was made in the dura and arachnoid membrane at the L3–L4 interlaminar space. A loop-type dialysis probe was then implanted. A PE10 catheter for the administration of saline or the local anesthetic to be tested was implanted intrathecally through the slit made at the L6–7 interlaminar space so that the tip of the catheter was located at the level of the cauda equina. The implanted dialysis probe was perfused with artificial cerebrospinal fluid bubbled with 95% oxygen and 5% CO₂ at pH 7.2. Samples were collected before and after administration of test substance and analyzed for glutamate.

After collecting the last sample (90 min after intrathecal administration of test substance) the catheters were removed, and all incisions were sutured. Isoflurane was discontinued, and the lungs were ventilated with 100% oxygen. Extubation of the trachea was

performed when adequate spontaneous ventilation occurred. The animals were allowed to recover with infusion of Ringer's solution and antibiotic treatment.

The animals were neurologically assessed daily until 1 week after test drug administration by an observer unaware of the treatment group. Sensory function was evaluated by seeking an aversive response to pinprick stimulation with a 23-gauge needle, progressing from sacral to thoracic dermatomes. The score of the sensory function was assessed by a three-point grading scale. The hind limb motor function was assessed by a five-point grading scale.

After completion of the neurologic function scoring at 1 week, the animals were re-anesthetized, and transcardiac perfusion and fixation were performed. The spinal cord was removed and refrigerated in phosphate-buffered formalin 10% for 48 h. After dehydration in graded concentrations of ethanol and butanol, the spinal cord was embedded in paraffin. The coronal sections of the spinal cord at L3, L4, and L5 levels were cut at a thickness of 8 μ m and stained with hematoxylin and eosin. The degree of spinal cord damage was assessed in terms of vacuolation of the dorsal funiculus with a four-point grading scale and chromatolytic changes in the motor neuron. The neurons with chromatolytic appearance were identified by round-shaped cytoplasm with the loss of Nissl substance from the central part of the cell and eccentric nuclei. The number of motor neurons with a chromatolytic appearance was counted in two sections for each animal and averaged.

Parametric data were presented as mean \pm SD. To determine differences in glutamate concentrations, a repeated-measures analysis of variance was performed. The cutaneous sensation, hind limb motor function, and morphological changes of the spinal cord were analyzed with a non-parametric method (Kruskal-Wallis test) followed by the Mann-Whitney *U*-test

Studies in Dogs

Wagner et al. (1940) performed spinal anesthesia in anesthetized **dogs** by puncture at the fourth lumbar interspace and measured the duration of anesthesia after the application of various local anesthetics.

Muschaweck et al. (1971) performed comparative intrathecal tolerance studies in dogs. **Beagle dogs** weighing 8–12 kg were anesthetized with 30 mg/kg sodium pentobarbital intravenously. The animals were intubated and submitted to artificial respiration. The fur on the neck was shaved and the skin disinfected. All further procedures were carried out under sterile

conditions. The spinal canal was punctured through the foramen magnum at the atlanto-occipital joint. Successful entry of the spinal cervical canal was checked by withdrawal of cerebrospinal fluid. Then 5 ml of cerebrospinal fluid was withdrawn and used as the solvent for the tested local anesthetics. The same volume was injected intrathecally either as a solution of local anesthetics in concentrations used in therapy or as control (saline solution). Artificial respiration was continued until spontaneous breathing resumed. Motor performance was checked for 24 h. Two days later, the animals were sacrificed under anesthesia. Dissection included the cerebellum, medulla oblongata, and sections from the cervical, thoracic and lumbar cord, carefully observing that the same segments were always taken, including the injection site. Segments were semi-fixed in 8% buffered formaldehyde for 2 days. When semi-fixed, the vertebral arches were opened, and the spinal cord as well as the roots of the spinal nerves were removed and completely fixed. After embedding in gelatin and Paraplast, a fast red 7B and myelin sheath staining according to Olivecrona were performed on serial sections from the site of injection as well as from cervical, thoracic and lumbar marrow. Hematoxylin–eosin staining as well as periodic acid-Schiff (PAS) staining and the iron reaction were all performed in one section. The presence or absence of nerve damage and of inflammation was noted.

A chronic model for investigation of experimental spinal anesthesia in the **dog** was described by Feldman and Covino (1981).

Dohi et al. (1987) inserted a polyethylene catheter into the lumbar subarachnoid space in **dogs** through a small hole in the dura for administration of drugs. The tip of the catheter was placed approximately 2–3 cm cephalad to the lumbar electrode introduced for recording of hydrogen clearance to measure spinal cord blood flow.

Kozody et al. (1985) measured spinal cord and spinal dural blood flow in the cervical, thoracic and lumbosacral regions in **dogs** using the microsphere technique. Measurements were taken 20 and 40 min after lumbar subarachnoidal injection through the L5-L6 or L6-L7 interspace.

Yaksh et al. (1995) studied the safety of chronically administered neostigmine methylsulfate in rats and **dogs**. Adult beagle dogs weighing 13–17 kg were adapted for 5 days to experimental protocols and placement of a nylon vest. For placement of the spinal catheter, the dogs were sedated (atropine 0.04 mg/kg and xylazine-Rompun 1–2 mg/kg i.m.) given an i.m. injection of penicillin G and procaine. Anesthesia

was induced by mask administration of halothane (3%–5%) and then the trachea was intubated. The dog was maintained under spontaneous ventilation with 1%–2% halothane and 50% N₂/50% O₂. Surgical areas on the back of the neck and head were shaved and prepared with alcohol and a povidone-iodine scrub, and the dog was placed in a stereotaxic head holder. After draping and using sterile technique, the cisterna magna was exposed, and a small incision (1–2 mm) was made. The intrathecal catheter (polyethylene tubing PE-50 stretched by 30%, making the nominal diameter 0.6 mm) was inserted and passed caudally at a distance of 40 cm, to a level corresponding approximately to the L3–L4 segment. The presence of the catheter in the intrathecal space was confirmed by free withdrawal of cerebrospinal fluid. A small stainless steel screw was placed in the skull and the catheter fixed to the screw. The catheter was tunneled subcutaneously and caudally to exit on the upper left back at the level of the scapula. The incision was closed by sutures, the halothane turned off and the animal allowed to recover. An analgesic was administered for postoperative pain medication. At this time, the catheter was connected to the infusion pump placed into a vest side pocket, and an infusion of sterile saline (2 ml/day) was started.

For a 28-day infusion study, dogs were randomly assigned to receive saline or neostigmine (4 mg/4 ml). After 28 days of infusion, the dogs were sacrificed. After induction of deep anesthesia, the animal was manually ventilated to maintain adequate oxygenation. A percutaneous puncture of the cisterna magna was performed and cerebrospinal fluid withdrawn for analysis. The chest was opened and a large-bore cannula placed in the aortic arch through which was perfused saline followed by 10% formalin. After fixation, the dura was exposed by an extensive laminectomy of the spinal canal and the lower brainstem, being careful to leave the catheter and the dura undisturbed. Dye was injected through the catheter to determine its integrity, visualize the position of the intrathecal catheter, and determine the spread of dye around the catheter. The spinal cord was removed in four blocks (cervical, thoracic, caudal, and rostral from the catheter tip), taking care to keep the dura intact, and placed in formalin. After fixation, tissue blocks were embedded in paraffin and then decalcified overnight, embedded in paraffin, sectioned at a thickness of 6–7 µm and stained with hematoxylin and eosin. Particular attention was given to the presence or absence of fibrosis and other reactions around the catheter, dural thickening or other reaction, such as inflammation in

the epidural space, leptomeninges/subarachnoid space, or spinal cord parenchyma, microglial nodules, demyelination, or gliosis. The degree of chronic and/or acute inflammation was graded as normal, mild, moderate or severe.

Studies in Other Species

Bahar et al. (1984b) performed chronic implantations of nylon catheters into the subarachnoid space of Wistar rats and marmosets and tested the effects of local anesthetics.

The technique of evaluation of spinal anesthesia by a local anesthetic in the **Rhesus monkey** was described by Denson et al. (1981).

Lebeaux (1975) recommended the **sheep** as a model for testing spinal and epidural anesthetic agents. To ensure accurate and reproducible administration of drugs into these restricted anatomical regions, a wooden stock was built to restrain the sheep without stress but to allow arching of the lumbosacral spine in order to open the inter vertebral space. The stock consisted of a table with holes for the front feet, hinged at one end to a fixed stanchion. The hinges permitted tilt of the table. The sheep was placed prone on the table, the head secured in the stanchion and the front feet passed through the holes and tied. The lumbosacral interspace used for both spinal and epidural tap was separated by positioning and tying the rear limbs cephalad beside the abdomen, thereby extending the knee and flexing the hip. The wool was clipped from the sheep's back, the skin of the lumbosacral area was washed and swabbed with iodine, and the vertebral spines and interspaces identified by palpation, marked, and numbered with a felt pen.

Spinal block was performed using either the midline or lateral approach for tapping the subarachnoid space. A 22-gauge disposable spinal needle was inserted subarachnoidally, and proper position was verified by spontaneous flow of cerebrospinal fluid upon removal of the stylet. With the table top and sheep parallel to the floor, the Luer-Lok syringe holding 1 ml of sterile 5.0% dextrose solution containing 2.5 mg (0.25%) tetracaine was attached to the needle. After aspiration of spinal fluid, the solution was injected within 5 s. At completion of the injection, spinal fluid was again aspirated to confirm injection into the subarachnoid space. The needle was withdrawn and the table tilted 10° caudally to restrict the spread of the hyperbaric solution to the lumbosacral area. The sheep was held in this position for 20 min.

Immediately after injection, the rear feet were untied and allowed to hang free on either side of the table

for evaluation of sensation and/or reflexes in the rear limbs. Then, 20 min after injection the sheep was removed from the table and positioned in a sling fashioned from a heavy duty 50-gallon plastic barrel split in half longitudinally and mounted on a metal support. Holes cut in the barrel allowed the feet to drop through so that comfortable sternal recumbency was maintained while permitting anesthetic evaluation to continue. When complete sensation returned in the skin of the dorsum, the sheep was removed from the sling and placed on a straw-bedded floor so that weight support and full recovery could be assessed.

Kyles et al. (1992) described a simple and noninvasive method for the chronic implantation of intrathecal catheters in the **sheep**.

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G.1.5.2

Blockade of Urethral Reflex in Rabbits

PURPOSE AND RATIONALE

For evaluation of spinal anesthetics the test substance has to be injected into the spine. For the study of local anesthetics for which conduction anesthesia has been verified, intraspinal injection provides more complete information. Spinal anesthesia has been studied in rabbits (Bieter et al. 1936a, 1936b; Luduena and Hoppe 1951; Luduena 1957; Gonzalez and Luduena 1961; Turner RA 1965), cats (Sechzer 1965), and dogs (Dvorak and Manson 1930). If the solution of test substance is injected intrathecally to male rabbits, the presence of anesthesia may be determined by the urethral reflex (Luduena 1957).

PROCEDURE

Male Chinchilla rabbits weighing 3.0–3.5 kg are used. The volume administered intrathecally is 0.02 ml per centimeter of spinal length. It is injected at a rate of 1 ml per min with a 22-gauge needle that is 3.8 cm long. The needle is introduced between the sixth and seventh lumbar vertebrae, and not through the lumbosacral space. The needle, held as lightly as possible between the thumb and forefinger, is introduced slowly until a typical sharp and sudden twitch occurs. This indicates penetration into the subarachnoid space. A one ml tuberculin syringe is then attached to the needle. It is not possible to aspirate fluid after insertion of the needle. However, it is possible to aspirate a portion of the injected solution. Very little pressure is required for injection of 0.4 to 0.6 ml of anesthetic solution while the animal is restrained in a canvas hammock. A catheter is inserted into the urethra, where it is kept without digital pressure. At 15- or 20-s intervals, 2 to 3 ml of water, at room temperature, are injected rapidly into the catheter. This causes the “urethral reflex”, consisting of retraction of the penis and contraction of the anal sphincter. The water runs out of the urethra around the catheter. Without medium or high concentrations of the anesthetics the reflex is absent

on the first reading. After loss of the urethral reflex, the test is repeated every minute or two for 15 min, and thereafter at longer intervals. The duration of urethral areflexia is taken as the time of the first positive reading (reflex absent) to the middle of the interval between the last positive and the first negative reading. This duration varies linearly with the logarithm of concentration.

EVALUATION

The threshold anesthetic concentration in grams per 100 ml for abolition of the urethral reflex for 5 min, the TAC₅, is a standard for comparison. There is a correlation between activity and systemic toxicity. The ratio of the activity to the irritancy is the most important parameter.

MODIFICATIONS OF THE METHOD

Burdyga and Magura (1986) studied the effects of local anesthetics on the electrical and mechanical activity of the guinea-pig ureter.

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G.1.6

Endoanesthetic Effect

PURPOSE AND RATIONALE

Endoanesthesia has been described as the effect of local anesthetics on visceromotor afferent receptors, located predominantly in the lung as distension receptors (Meier and Bein 1950; Zipf 1953, 1957, 1959, 1966.

1968; Zipf et al. 1955; Zipf and Oehler 1955; Reichertz et al. 1957; Zipf and Reichertz 1957; Zipf et al. 1963; Wellhöner and Conrad 1965; Siemoneit et al. 1966; Dittmann and Zipf 1973; Borchard 1979). The endoanesthetic effect has been claimed to be the basis of an antitussive action of local anesthetics (Bucher 1956; Bein and Bucher 1957; Kraushaar et al. 1964).

PROCEDURE

Male guinea pigs weighing 300–400 g are anesthetized by intraperitoneal injection of 1.25 g/kg urethane. The studies are carried out on spontaneously breathing or artificially respired monovagotomized or bivagotomized animals. Intravenous injections are given via a catheter into the jugular vein. The flow rate of the respiratory air is measured with a Fleisch tube connected to a pneumotachograph. In experiments with positive pressure respiration, the open end of the Fleisch tube is attached to a respiratory pump (40 strokes per min). A cannula is inserted into the carotid artery and connected to a pressure transducer.

The left nervus vagus is exposed from the thorax aperture until his entry into the foramen jugulare and cut in the middle of the distance. Both ends are placed over 3 wires of Ag/AgCl 6-way electrodes. The edges of the skin wound are pulled upwards with clamps and the space around the trachea is filled with paraffin to prevent the nerve from drying out. The afferent and efferent vagus potentials and also their integration curve (Zipf and Reichertz 1957) are recorded on a direct recorder. In addition, the ECG in the second lead is recorded continuously. The animal is shielded electrically with the aid of a Faraday cage.

The nervus vagus conducts not only afferences from the lung but also from other organs to the brain. The base points of the integral curve, which correspond to the vagus action potentials being synchronous with inspiration and issued by the pulmonary distension receptors, therefore lie above the baseline of the recorder tracing. The vagus afferences which remain after subtraction of the vagus activity synchronous with respiration ("inspiratorial activity") are called "residual activity". The level of inspiratorial activity depends both on the bronchial width (degree of inflation of the lungs) and on the functional state of the pulmonary receptors and of the afferent pathway. The occurrence of a bronchospasm is detectable from the decrease in the pneumotachogram, if the respiratory rate is not reduced at the same time.

EVALUATION

The following parameters are evaluated:

Afferent and efferent inspiratorial vagal activity, maximum flow rate of the respiratory air during inspiration and expiration, tidal volume, duration of the respiratory phases (inspiration = t_i , expiration = t_e , respiratory pause = t_p), heart rate and blood pressure.

The decrease of electrical activity after various doses of standard and test drugs is calculated at different time intervals after drug administration. Maximum of activity and decay with time are registered.

Statistical analysis is performed using the paired and non-paired *t*-test, after the applicability has been first checked with the F-test.

CRITICAL ASSESSMENT OF THE METHOD

The correlation between antitussive and local anesthetic activity has been challenged by Sell et al. (1958) and by Ther and Lindner (1961).

MODIFICATIONS OF THE METHOD

Bein and Bucher (1967), Wellhöner and Conrad (1955) tested various compounds for their anesthetic effect on pulmonary stretch receptors of **rabbits**.

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G.1.7

Effect on Electroretinogram

PURPOSE AND RATIONALE

The retina, which belongs in ontogenesis to the central nervous system, can be prepared without damaging the parenchyma. The transretinal potential which can be induced by a light flash can be used as an objective criterion for the functional state (Sickel et al. 1960). The multi-phase potential path of the electroretinogram consists of an initial a-wave, a subsequent b-wave and an e-wave which starts with a delay after the end of the stimulus. While the amplitudes of the a- and e-wave are in the order of 50 μV , the amplitude of the b-wave (Φ_b) is 500–1000 μV . The electroretinogram can be used for various purposes, such as the evaluation of local anesthetics (Borchard 1979).

PROCEDURE

Frogs (*Rana esculenta*) are kept in dark for 1 h before beginning of the preparation. The frog is sacrificed by decapitation, the eyeball excised in dim red light and cut into two halves with the aid of a rotating blade. The retina together with the pigmented epithelium and choroid is removed from the rear half of the eye and

transferred to a dish with Tyrode solution. The retina is then detached from the pigmented epithelium by careful shaking and spread out on a Monodur net. The net is fixed to a round carrier with a narrow ring and the chamber is transferred to a flow-through apparatus. An electrical pump is used for superfusion of the retina with Tyrode solution.

In order to apply the local anesthetic only to the receptor or vitreous body side of the retina, an apparatus is used in which the tissue on a silk net is stretched as the partition between two chamber halves which can perfused separately (Borchard and Erasmi 1974).

The electroretinogram is recorded with Ag/AgCl electrodes. After amplification, the signal is registered on a recorder or an oscillograph. Light stimuli of 10 ms are generated. The light from a low-voltage light source is focused on a electromagnetic shutter with a stimulus frequency of 0.1 Hz. Light stimuli of 1 s duration are used at intervals of 3 min to measure the influence of the local anesthetics on the electroretinogram as a function of light intensity.

All experiments are performed after adaptation to darkness and at room temperature. The amplitude of the b-waves (Φ_b) which is measured from the lowest point of the a-wave to the maximum of the b-wave is used for evaluation. In order to obtain stationary test conditions, the experiment is started after a 45 min adaptation period. The stimulus signal is recorded by means of a photocell inserted in the light path.

Various concentrations of the local anesthetics are applied and various light intensities are used. The decrease of the b-wave starts immediately and then progresses slowly and continuously. After changing over to Tyrode solution, the decrease in the exposure potential is completely reversible.

EVALUATION

After various doses, time-response curves can be drawn and for a given time interval ED_{50} values can be calculated. Moreover, the influence of local anesthetics on the b-wave at various light intensities can be estimated.

MODIFICATIONS OF THE METHOD

Isolated retina for electroretinogram has been used not only from **frogs** but also from **rats** (Huang et al. 1991; Doly et al. 1993), **rabbits** (Mochizuki et al. 1992; Maynard et al. 1998) and from **beef** (Gosbell et al. 1996; Walter et al. 1999).

Moreover, the electroretinogram has been used as parameter for many *in vivo* studies with different purposes, e.g., on the effect of drugs after retinal ischemia, in various animal species, such as **rats** (Sugimoto et al. 1994; Hotta et al. 1997; Biró et al. 1998; Block and Schwarz 1998; Estrade et al. 1998; Ettaiche et al. 1999; Li et al. 1999), **rabbits** (Takei et al. 1993; Zemel et al. 1995; Horiguchi et al. 1998; Jarkman et al. 1998; Liang et al. 1998), **cats** (Imai et al. 1991; Ostwald et al. 1997; Kim et al. 1998), **dogs** (Jones et al. 1995; Yanase and Ogawa 1997) or **monkeys** (Tagliati et al. 1994).

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G.2 Neuromuscular Blocking Activity

G.2.0.1 General Considerations

Neuromuscular transmission is mediated by mediated by nicotinic acetylcholine receptors for which various

subtypes are described (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

Neuromuscular blocking agents are distinguished by whether or not they cause depolarization of the motor end plate. They are classified either as competitive (stabilizing) agents, of which d-tubocurarine is the classical example, or as depolarizing, desensitizing agents such as succinylcholine.

The **rabbit head-drop method** was described by Varney et al. (1948, 1949), Burn et al. (1952), Levis et al. (1953). Rabbits were given an interrupted intravenous injection at a rate of 0.1 ml every 15 s until the muscles supporting the head become sufficiently relaxed to prevent the head to be raised when the back is stimulated. This method has been replaced by the rabbit sciatic nerve-gastrocnemius muscle preparation, the isolated phrenic nerve diaphragm preparation of the rat, and the chick sciatic nerve-tibialis anticus muscle preparation.

The method described by Allmark and Bachinski (1949) used an inclined screen for testing curare-like activity in **rats**.

Skinner and Young (1947) placed **mice** weighing 15–17 g in sloping rotating cylinders. Mice falling away during 20 min after subcutaneous injection were considered as reactors. Dose response-curves and potency ratios could be calculated from the logarithmic dose-response curves using different doses of test compound and the standard tubocurarine chloride.

Collier et al. (1949) used a rotating drum to assess the activities of paralyzant, convulsant and anesthetic drugs in mice.

Fatt and Katz (1951) performed an extensive study on neuromuscular junction by recording end-plate potentials from curarized frog sartorius motor end-plates, using a KCl-filled microelectrode inserted into the muscle fiber in the region of the motor end-plate.

Electrophysiological analysis of transmission at the skeletal neuromuscular junction was reviewed by Prior et al. (1993).

A review on new neuromuscular blocking drugs was given by Hunter (1995).

Savarese et al. (1975) discussed the potential uses of short-acting non-depolarizing neuromuscular blocking agents as predicted from animal experiments.

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G.2.0.2

Isolated Phrenic Nerve Diaphragm Preparation of the Rat

PURPOSE AND RATIONALE

The isolated phrenic nerve diaphragm preparation of the rat was originally described by Bülbbring (1946) to study the influence of adrenaline on tissue functions normally elicited by acetylcholine. The method has been modified and is widely used by many investigators for studying drugs affecting the neuromuscular transmission.

PROCEDURE

Adult male Wistar rats are used. The animal is sacrificed and the blood is drained. The skin is removed from the middle of the chest. The thorax is opened and the front part of the left thoracic wall is removed.

The phrenic nerve can be seen quite distinctly. The nerve is cut just below the thymus and a thread is attached to the cut end. The nerve is then freed carefully from the attached tissue. However, no attempt is made to clean the nerve completely from all attached tissue. An incision is made in the left abdominal wall just below the diaphragm. Two converging cuts are made through the diaphragm and the ribs towards the tendinous part of the diaphragm with the phrenic nerve attached to the center of the diaphragm. The fan-like preparation is about 3 mm wide at the tendinous end and is about 15 mm wide at the costal margin. A thread is attached to the tendinous part of the diaphragm.

The preparation is fixed by a stainless steel rod with a pair of pins hooked to the rib. It is lowered into an organ bath and the thread from the muscle is attached to a force transducer. The nerve is stimulated with a pair of electrodes with a hole about 1 mm wide. The right phrenic nerve-diaphragm preparation is isolated in the same manner. The organ bath containing Tyrode solution with 2.0 g/l glucose is oxygenated at 37°C with 95% O₂ and 5% CO₂. The nerve is stimulated 12 times per min by rectangular-wave pulses of 0.5 ms duration at 3–5 V. Contractions are isometrically recorded through a transducer on a polygraph. The test drugs are left in the organ bath either for short periods of time (3–8 min) or for as long as the maximum effect can be observed. After a wash-out period of 3–5 min, the next dose can be added.

EVALUATION

The force of contractions after addition of various doses of the test drug is compared with the effect seen prior to drug application. Dose-response curves can be established.

CRITICAL ASSESSMENT OF THE METHOD

The isolated phrenic nerve diaphragm preparation of the rat is an excellent method for determining the potency of a drug to block or facilitate neuromuscular transmission. However, it is not a good preparation for differentiating between depolarizing and non-depolarizing neuromuscular blocking agents because, in many cases, depolarizing blocking agents fail to demonstrate initial facilitation and fail to reverse the effect of non-depolarizing blocking agents. For differentiating between depolarizing and non-depolarizing blocking agents the chick sciatic nerve-tibialis anticus muscle preparation is preferred. In this preparation, the curare-like drugs produce a neuromuscular blockade whereas the decamethonium-like drugs in-

duce a contracture of the slow fiber when the neuromuscular transmission is blocked.

MODIFICATIONS OF THE METHOD

Colbert et al. (1990) studied the effects of temperature on the experimental reliability of the isolated rat phrenic nerve/diaphragm preparation.

Vizi et al. (2003) used *in vitro* isolated phrenic nerve-hemidiaphragm preparations of mice, rats and guinea pigs and *in vivo* sciatic nerve-anterior tibialis muscle preparations from anesthetized rats, guinea pigs and cats to characterize a new short-acting non-depolarizing muscle relaxant.

Van Riezen (1968a, b) described the sciatic nerve-tibialis anticus muscle preparation in **chicks**. Three to 8 day old chicks are decapitated and the skin of the legs is rapidly removed. The leg is separated from the body by cutting through the hip joint. The muscles of the thigh are dissected and the sciatic nerve with the superficial peroneal branch is freed from the upper leg tissue. The fascia is removed from the lower leg and the tibialis anticus tendon identified. A thread is attached to the tendon of the muscle and the tibialis anticus muscle is freed towards but not up to the knee joint attachment where the nerve enters into this region. The upper and lower leg bones are then cut off leaving the muscle with its nerve attached to the knee joint. The tendon is fastened by a hook to a ring in the bottom of the organ bath. A thin steel rod is attached to the knee joint. The contractions of the muscle are recorded isometrically. The nerve is passed through an electrode similar to that used in the rat phrenic nerve-diaphragm method. The nerve is stimulated six times per min for 0.5 ms duration at supramaximal voltage.

Jenden et al. (1954) described the isolated lumbrical muscle of the **rabbit** as a preparation which is sensitive to competitive (*d*-tubocurarine-like) and depolarizing (decamethonium-like) blocking agents. This muscle is cylindrical in shape, about 18 mm long and 1 mm in diameter and has a wet weight of about 15 mg.

Jenden (1955) used the isolated diaphragm of **guinea pigs** to study the effect of drugs upon neuromuscular transmission.

Hoppe (1955) reviewed the potency of neuromuscular blocking agents in various species.

Birmingham and Hussain (1980) used the phrenic nerve-diaphragm and the hypogastric nerve-vas deferens preparation of the guinea pig for comparison of the skeletal neuromuscular and autonomic ganglion-blocking potencies of non-depolarizing relaxants.

Wessler and Kilbinger (1986), Wessler and Steinlein (1987), Wessler et al. (1992) described a modi-

fied **rat** phrenic nerve-hemidiaphragm preparation whereby most of the muscle was cut off (end-plate preparation).

Muir et al. (1989) used biventer cervicis nerve-muscle preparations from **young chickens** and phrenic nerve-hemidiaphragm preparations from **rats** to evaluate neuromuscular blocking agents. Micro-electrode recordings were obtained from the nerve-hemidiaphragm preparation of the rat and from the costocutaneous nerve-muscle preparation of the North American **garter snake** (*Thamnophis sirtalis*). The later preparation is particularly suitable for voltage clamp recording from the neuromuscular junction because it possesses large diameter fibers which aid visualization and penetration of endplates. This, coupled with compact endplates, allows good control of membrane voltage over the entire endplate region when using the two-microelectrode voltage clamp technique.

In order to record evoked endplate currents without accompanying muscle contraction, cut fiber preparations were used. Dissection and cutting of muscle fibers was performed at low K^+ (2 mmol/L) physiological solution perfused for approximately 30 min. Snake nerve-muscle preparations were mounted in a physiological salt solution of pH 7.1–7.2 containing (mmol/l) NaCl 159, KCl 4.2, $CaCl_2$ 1.5, $MgCl_2$ 4.2, HEPES 10. Rat nerve-muscle preparations were mounted in Krebs solution of the same composition as that used for tension experiments. The muscles were mounted in a Sylgard-coated Perspex dish and endplates were voltage clamped using glass capillary microelectrodes (resistance 2–10 M Ω). Voltage recording electrodes were filled with potassium chloride 3 mol/l and current passing electrodes were filled with potassium sulfate 0.6 mol/l.

The nerves were stimulated through platinum electrodes at a frequency of 0.5 Hz with rectangular pulses of 0.05 ms duration and of strength sufficient to produce evoked endplate currents. The evoked endplate currents were filtered by a 5-kHz low pass filter and recorded on magnetic tape. The currents were amplified and digitized by a laboratory interface connected to a computer at a digitization rate of 25 kHz. Ten to 20 evoked endplate currents were collected and averaged after alignment at the middle of their rising phase. Evoked endplate currents decayed as a single exponential function according to the following relationship:

$$I(t) = I(0) \exp^{-t/\tau}$$

where $I(t)$ is the current amplitude at time t after the peak, $I(0)$ is the peak current amplitude and τ is the decay constant.

Experiments were carried out at room temperature. Drug solutions were perfused through the tissue bath for 10 min by peristaltic pump, ensuring a complete exchange of solution.

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G.2.0.3

Sciatic Nerve-Gastrocnemius Muscle Preparation in the Rabbit

PURPOSE AND RATIONALE

Levis et al. (1953), Long and coworkers (1959, 1967, 1969) described the sciatic nerve-gastrocnemius muscle preparation in the rabbit as an *in vivo* model for testing neuromuscular blocking agents.

PROCEDURE

Dutch rabbits, weighing 1–2 kg, are anesthetized with 200 mg/kg of phenobarbital administered slowly into the marginal ear vein. The sciatic nerve is ligated and cut, and a shielded electrode is placed on the peripheral portion of the nerve. The gastrocnemius muscle is freed as completely as possible from surrounding muscles and a thread is attached to the tendon of the muscle. The twitches of the muscle are elicited by supramaximal stimulation and are recorded through a force transducer. The parameters for interrupted tetanic stimulation are 250 c.p.s. with pulse durations of 1 ms at 15 V applied for 0.2 s every 10 s. The test drugs are administered intravenously into the marginal ear vein.

EVALUATION

The force of contraction after injection of various doses of the test drug is compared to the values obtained prior drug administration.

CRITICAL ASSESSMENT OF THE METHOD

The preparation has the advantage of studying the drug effects under conditions similar to clinical use.

MODIFICATIONS OF THE METHOD

There are two other preparations, the **cat soleus muscle** preparation and the **cat tibialis anticus muscle**

preparation that very useful. They can be prepared in a method similar to that described for gastrocnemius muscle except that close arterial injection can be made using the tibialis anticus preparation. Also, soleus muscle consists primarily of slow muscle, whereas tibialis anticus is fast muscle. The experimental procedures for these two preparations are described in detail by Brown (1938), Bowman et al. (1962), Salafsky (1968).

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G.2.0.4

Evaluation of Neuromuscular Blockade in Cats, Pigs, Dogs and Monkeys

PURPOSE AND RATIONALE

Neuromuscular blocking drugs have been evaluated in various animal species. Muir et al. (1991) studied the effects of an analog of vecuronium in anesthetized cats, pigs, dogs and monkeys.

PROCEDURE**Anesthetized Cats**

Experiments were carried out on cats of either sex anesthetized with a mixture of α -chloralose (80 mg/kg) and pentobarbitone (5 mg/kg) injected intraperitoneally. Animals were ventilated with air at a rate of 26 breaths per minute using a tidal volume of 13 ml/kg. The right hind limb was immobilized and the contractile responses of the tibialis anterior and soleus muscles to single shock stimulation of the sciatic nerve were recorded. The sciatic nerve was stimulated at a rate of 0.1 Hz using rectangular pulses of 0.2 ms duration and of a strength greater than that required to produce a maximal twitch. Contractions of the nictitating membrane were evoked in response to preganglionic stimulation of the cervical sympathetic nerve with 10-s-duration trains at a frequency of 5 Hz and a strength sufficient to produce maximal contractions of the nictitating membrane. Arterial blood pressure was recorded from the carotid artery using a Statham PC45 pressure transducer. The blood pressure pulse triggered a cardiograph to display the heart rate. Both vagus nerves were ligated and, at 100-s intervals, the right vagus nerve was stimulated with 10-s-duration trains at a frequency of 2–5 Hz and with pulses of 0.5 ms duration and strength greater than that required to produce a maximal reduction of heart rate. Contractile responses of muscles were recorded using Grass FTO3C and FT10C force displacement transducers. All responses were displayed on a Grass model 5 ink writing oscillograph.

Savarese (1979) determined not only the potencies of metocurine and d-tubocurarine but also the autonomic margins of safety in anesthetized cats.

PROCEDURE

Adult cats of either sex are anesthetized with α -chloralose, 80 mg/kg, and pentobarbital 7 mg/kg, given intraperitoneally. Cannulas were placed in the left femoral vein and artery for drug injection and recording blood pressure and heart rate. The lungs were mechanically ventilated through a tracheostomy and a small animal ventilator set to deliver 15 ml/kg tidal volume and 20 breaths/min.

The right vagus nerve and the right sympathetic trunk were exposed and divided in the neck. The distal ends were placed on the same shielded platinum wire electrode to permit preganglionic stimulation of both nerve trunks. The left sympathetic trunk was also dissected along its postganglionic portion at the base of the skull, and cut distal to the superior cervical gan-

glion to permit postganglionic stimulation through another electrode. Trains of square wave pulses (20 Hz for 10 s) were delivered at supramaximal voltage every 4 min simultaneously to all three autonomic nerve trunks. The resulting bradycardia and hypotension (the vagal response) were measured. Contractions of both nictitating membranes, one (the right) elicited preganglionic and the other (the left) elicited postganglionic, were recorded. The maximal vagal response (i. e., cardiac arrest for 10 s) was achieved by stimulation of the right vagus nerve or of both vagus nerves.

Twitches of the right tibialis anterior muscle were elicited at 0.15 Hz via the peroneal branch of the right sciatic nerve, to which square-wave shocks of 0.2 ms were applied at supramaximal voltage. Twitch recording was done via a transducer. All nerves and tendons were kept moist in small pools of mineral oil or in cotton pledges soaked in mineral oil. Tibialis anterior and esophageal muscle temperatures were monitored and kept between 35°C and 38°C by heat lamps.

Simultaneous recordings of heart rate, arterial pressure, pre- and postganglionic elicited contractions of the nictitating membrane, and twitches of the tibialis anterior muscle were made on a polygraph. Cumulative dose–response curves for inhibition of neuromuscular, vagal (parasympathetic) and sympathetic functions were determined simultaneously for each animal. The mechanism of vagal inhibition was localized at parasympathetic ganglia or cardiac muscarinic receptors by determining whether the bradycardic response to methacholine (20 μ g/kg) was blocked as well as the neurally elicited bradycardia.

A single-bolus dose of the neuromuscular relaxants producing the delayed depressor response plus tachycardia (Paton 1957) was determined in each animal. This response, being pathognomonic for histamine release, is defined as sudden hypotension to less than 80% of the control arterial pressure within 2 min of relaxant injection and with tachycardia to more than 25% above the baseline value.

Test drugs were given intravenously.

EVALUATION

Data analysis was done by the method of Litchfield and Wilcoxon. Mean dose–response curves were plotted on log-probit paper. Best fit to straight lines on these scales was determined by computerized regression. The cumulative ED₅₀ values for vagal and sympathetic inhibition and the cumulative ED₉₅ values for neuromuscular blockade were determined from the lines and 95% confidence limits were calculated. Dif-

ferences in potency were considered significant when $P < 0.05$.

The occurrence of histamine release was also treated as an all-or-none response to permit log-probit plotting. The delayed depressor response plus tachycardia was judged to have or have not occurred after each single bolus injection of the drugs. The percentage of animals responding at each dose level was then determined and the data handled by the Litchfield–Wilcoxon method.

The autonomic margins of safety of the test drugs were calculated as the ratios of cumulative doses producing 50% block (ED_{50}) of vagal (parasympathetic) and sympathetic transmission and the ED_{50} for histamine release, each divided by the ED_{50} of neuromuscular blockade.

Anesthetized Dogs

Anesthesia was induced in beagle dogs using intravenous pentobarbitone sodium (3 mg/kg). Animals were intubated, without the use of a muscle relaxant, and artificially ventilated. Anesthesia was maintained with halothane (1.2% inspired concentration) and oxygen. The electrocardiogram was recorded continuously and the signal used to integrate heart rate throughout the experiment. Catheters were inserted percutaneously into the right femoral artery and vein for the recording of arterial blood pressure and the injection of drugs respectively.

A “boomerang” type transducer, originally constructed for recording adductor human pollicis tension (Waltz 1973), was fitted to the lower left leg for recording muscle contractions. Submaximal rectangular pulses of 0.1 ms duration at a frequency of 0.1 Hz were delivered to the sciatic nerve through electrodes inserted percutaneously, and the resultant twitches of the foot recorded.

Clutton et al. (1992) studied the autonomic and cardiovascular effects of neuromuscular blockade antagonism in the **dog**. Neuromuscular blockade was antagonized with various anticholinesterase-antimuscarinic drug combinations including atropine, neostigmine, and glycopyrrolate.

Anesthetized Pigs

Domestic pigs of either sex (10–15 kg) were administered a tranquillizing dose (approximately 2 mg/kg) of azaperone about 20 min before induction of anesthesia with 3%–4% halothane in oxygen. Following induction, anesthesia was maintained with α -chloralose (200 mg/kg) dissolved in polyethylene glycol 300 given slowly into a jugular vein. Approximately 1

h later, anesthesia was supplemented with additional chloralose given by slow intravenous infusion which was continued throughout the investigation. The lungs were mechanically ventilated with room air via a tracheal cannula at a rate of 28 breaths per minute and a tidal volume of 12–14 ml/kg. Arterial pressure was recorded through a polyethylene catheter placed in the right carotid artery and connected to a Gould–Statham pressure transducer. Heart rate was monitored continuously by using the arterial pulse pressure to trigger a Grass 7P4F cardiometer. Drugs were administered through a catheter in the contralateral vein. Contractions of the tibialis anterior and soleus muscle were recorded by force displacement transducers. A resting tension of 30 g was applied to each muscle. Twitches were evoked every 10 s by stimulation the two branches of the sciatic nerve supplying the lower leg, immediately distal to the point where the nerve divides, using square wave pulses of 0.25 ms duration and at twice the voltage required to produce maximum contraction.

Anesthetized Rhesus Monkeys

Rhesus monkeys were anesthetized with intramuscular ketamine (10 mg/kg). Endotracheal intubation was performed without the use of muscle relaxants. Anesthesia was maintained using a single bolus of pentobarbitone sodium (4 mg/kg) given intravenously followed by infusion, which was adjusted to maintain a steady state of anesthesia. Heart rate was measured continuously by integration of the electrocardiogram. Atropine sulfate (0.25–0.5 mg) was administered i.m. to prevent excessive salivation. Artificial ventilation was supplied and blood pressure recorded continuously. The ulnar nerve was stimulated with rectangular electrical pulses via bipolar subcutaneous needle electrodes. The twitch response of the adductor pollicis muscle were recorded via a Statham transducer attached by a wire to a small U-clamp, which was fixed firmly to the basal phalanx of the thumb.

EVALUATION

Experiments were performed in the various animal species by injecting different doses at hourly intervals to obtain a range of neuromuscular paralysis, i. e., between 10 and 95% inhibition of single-twitch tension. From the data, dose-inhibition lines were constructed using the Levenberg-Marquardt non-linear interactive curve-fitting routine (Brown and Dennis 1972). From these data, doses producing 50% inhibition of induced contractions of the tibialis anterior muscle (cat and pig), hind limb (dog) and adductor pollicis muscle

(monkey) were calculated. Time course measurements were made with doses which produced between 85% and 95% neuromuscular block. Onset time was measured as the time from injection to the first maximally depressed contraction. The recovery time was the time from 75% block to 25% block and the duration of action was the time elapsing from injection to 90% spontaneous recovery compared to pre-drug control twitch tension.

MODIFICATIONS OF THE METHOD

Bowman et al. (1988) investigated structure:action relationships among some desacetoxo analogues of pancuronium and vecuronium in the anesthetized **cat**. Blockade of sciatic nerve-induced contraction of the tibialis and soleus muscles, as well as the effects on vagal-induced bradycardia and on sympathetically induced contractions of the nictitating membrane, were studied.

Khuenl-Brady et al. (1990) used anesthetized **cats** to study the effects of two new nondepolarizing neuromuscular blocking drugs. The indirectly evoked twitch tension of the anterior tibialis muscle elicited by supramaximal square-wave stimuli applied to the peroneal nerve was continuously quantitated by means of a force-displacement transducer and recorded. Onset time (from injection of muscle relaxant to maximum depression of twitch tension), duration of action and recovery index were determined.

Muir et al. (1989) used tibialis anterior and soleus muscle/sciatic nerve preparations in anesthetized **cats** and **pigs** to evaluate neuromuscular blocking agents.

Hoppe (1950) used a nerve-muscle preparation in **dogs** to evaluate curarimimetic drugs. Diaphragmatic respiration was recorded directly by means of a light thread from a suture imbedded in the peritoneal aspect of the right hemi-diaphragm. Stimulation of the peripheral end of the sectioned tibial nerve was accomplished by an induced current once every ten seconds. Muscle contraction was recorded from the Achilles tendon being severed just proximal to the calcaneus.

Hughes and Chapple (1976a, b, 1981; Hughes 1984) used nerve-muscle preparations of **cats**, **dogs** and **rhesus monkeys**.

Ono et al. (1990) recorded the twitch tension of the gastrocnemius-soleus muscle in **rats** after stimulation of the distal stump of the tibial nerve under the influence of a centrally acting muscle relaxant.

Cullen et al. (1980) described two mechanical techniques to measure neuromuscular activity in the intact, anesthetized **dog**. Simultaneous stimulation of the dor-

sal buccal branch of the facial nerve and ulnar nerve was performed and the evoked mechanical muscle responses measured.

Keeseey (1988) discussed the use of single-fiber electromyography (SFEMG) by the AAEE minimonograph 33 as an electrodiagnostic approach to defects of neuromuscular transmission.

Marshall et al. (1994) gave an overview of the pharmacology of rocuronium bromide in experimental animals.

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G.2.0.5

Evaluation of Neuromuscular Blockade in Anesthetized Mice

PURPOSE AND RATIONALE

Electromyographic investigations of the neuromuscular junction are relatively easy to perform in large animal species such as the dog (Cullen et al. 1980). A simple *in vivo* method for the quantitative evaluation of neuromuscular blockade in anesthetized mice has been described by Lefebvre et al. (1992).

PROCEDURE

Female BALB/c mice, 7–9 weeks old, are anesthetized by intraperitoneal administration of 50 mg/kg etomidate or 250 mg/kg mephenesin. Hair is removed from the sciatic area and hind leg by application of a depilatory cream. A lamp is placed 30 cm above the anesthetized animal to maintain a constant body temperature. Two monopolar needle-stimulating electrodes are subcutaneously inserted into the sciatic notch area and two monopolar recording electrodes subcutaneously over the gastrocnemius belly and in the vicinity of the tendo calcaneus communis, respectively. A ground electrode is inserted under the skin between recording and stimulating electrodes. Stimulation is carried out using a square signal of 0.2-ms duration. The supra-maximal stimulation of the sciatic nerve consists of a train of 10 stimuli, lasting 3.3 s with a frequency of 3 Hz. Stimulation intensity is fixed to a value 50% higher than that required to attain the maximal evoked potential response. Two control trains of stimulation are applied before test-drugs administration and then repeated at 1-min intervals until the end of anesthesia which is assessed by reflex movement after pinching the toes or the tail.

Muscle action potentials are recorded using an electromyograph. The low and high cut-off frequencies are set to 16 Hz and 10 kHz, respectively. The electromyogram signal is amplified and plotted on an oscilloscope screen and printed. Evoked active potentials are analyzed using an 8 bits digitizer and the area under the response wave is evaluated for each stimulus (S).

The neuromuscular blocking agents are administered by intraperitoneal route.

EVALUATION

The effects of the test drugs are quantified by measuring the ratio of the fifth response (S₅) to the first one (S₁) in a given train (S₅/S₁ response) according to Keeseey (1989). These values are almost not influenced by etomidate anesthesia. Neuroblocking agents of competitive type, such as alcuronium, or depolarizing type, such as suxamethonium, decrease this ratio significantly. These effects can be blocked by neostigmine, but not the effect of the snake venom alpha-bungarotoxin.

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G.3 Safety Pharmacology

See Vogel (2006).

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Chapter H

Analgesic, Anti-inflammatory, and Anti-pyretic Activity¹

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¹Contributions to earlier editions by R. Schleyerbach, K.U. Weithmann and R.R. Bartlett.

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| H.3.1.8.3 | Effect of TNF- α Binding | 1084 | | Pain is a symptom of many diseases requiring treat- ment with analgesics. Severe pain due to cancer metas- tases needs the use of strong analgesics, that means opioid drugs. The addiction liability of opioids led to intensive research for compounds without this side ef- fect. Many approaches have been used to differentiate the various actions of strong analgesics by developing animal models not only for analgesic activity but also | |
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for addiction liability. Several types of opioid receptors have been identified in the brain allowing *in vitro* binding tests. However, the *in vitro* tests can only partially substitute for animal experiments involving pain. Pain is a common phenomenon in all animals, at least in vertebral animals, similar to that felt by man. Analgesic effects in animals are comparable with the therapeutic effects in man. Needless to say, that in every instance painful stimuli to animals must be restricted as much as possible. Painful stimuli can consist of direct stimulation of the efferent sensory nerves or stimulation of pain receptors by various means such as heat or pressure. The role of endogenous peptides such as enkephalins and endorphins gives more insight into brain processes and the action of central analgesics.

Pain can also be elicited by inflammation. Progress has been made in elucidating the role of various endogenous substances such as prostaglandins and peptides in the inflammatory process. Most of the so called non-steroidal anti-inflammatory agents have also analgesic activity. Lim and Guzman (1968) differentiated between antipyretic analgesics causing analgesia by blocking impulse generation at pain receptors in the periphery while the narcotic analgesics block synaptic transmission of impulses signaling pain in the central nervous system. An old but excellent survey on methods being used to test compounds for analgesic activity has been provided by Collier (1964). Today, the classification into central and peripheral analgesics is definitively too simplified (Bannwarth et al. 1993) but provides a guide for differentiation by pharmacological methods.

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H.1.1

In Vitro Methods for Central Analgesic Activity

H.1.1.1

Survey

In 1973, high-affinity stereospecific binding of radio-labeled opiate compounds by CNS membrane prepa-

rations was reported (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). The *in vivo* pharmacological potency of opiate agonists and antagonists parallels the *in vitro* displacement of ^3H -naloxone, a potent narcotic antagonist. Based on these findings, the ^3H -naloxone binding assay was introduced for evaluation of potential analgesics with opiate-like properties. According to different pharmacological profiles of opiates, several receptor types have been identified designated as μ , κ , δ , and σ receptor (μ for morphine = MOP receptor, κ for ketocyclazocine = KOP receptor, δ for deferens because it was first identified in mouse vas deferens = DOP receptor). The σ receptor (σ for SKF10047) was only initially classified as an opioid receptor (see below). Several reviews on opioid receptors have been published: Knappe et al. (1995), Mansour et al. (1995), Satoh and Minami (1995), Dhawan et al. (1996), Singh et al. (1997), Standifer and Pasternak (1997), Law et al. (2000), Snyder and Pasternak (2003), Janecka et al. (2004), Eguchi (2004), and Waldhoer et al. (2004).

The opioid receptors were reclassified according to recommendations of the International Union of Physiological Sciences, the International Union of Pharmacology (IUPHAR; Dhawan et al. 1996, 1998; Alexander and Peters 2000). This nomenclature applies an abbreviation of the generic term for the family (OP for opioid) and a subscript number. **OP₁ stands for δ , OP₂ for κ , and OP₃ for μ receptor.**

For the μ receptor, subtypes named μ_1 and μ_2 have been described (Fowler and Fraser 1994; Traynor 1994; Pasternak 2001). Analgesia is thought to involve activation of μ receptors (largely at supraspinal sites) and κ receptors (principally within the spinal cord); δ receptors may also be involved at the spinal and supraspinal level. Other consequences of μ activation include respiratory depression, miosis, reduced gastrointestinal motility, and euphoria. The μ_1 receptors are postulated to mediate the supraspinal analgesic action and the μ_2 receptors to mediate respiratory depression and suppression of gastrointestinal motility. Moreover, different effects on heart rate were described (Paakkari et al. 1992). Two endogenous peptides were described, named **endomorphins**, as agonists with high specific affinity for the μ -receptor (Hackler et al. 1997; Zadina et al. 1997, 1999; Horvath 2000).

Several studies provide evidence for the existence of **δ -opioid receptor subtypes** (Sofuoglu et al. 1991; Porreca et al. 1992; Horan et al. 1993; Miyamoto et al. 1993; Tiseo and Yaksh 1993; Burkey et al. 1998).

Binding studies with δ opioid receptors have been performed by Mosberg et al. (1983). Simonin et al. (1994) reported the genomic organization, cDNA cloning, the functional expression in COS cells, and the distribution in human brain of the human δ -opioid receptor.

Endogenous ligands for δ receptors are **enkephalins**.

A rat κ **opioid receptor** has been cloned (Meng et al. 1993). Evidence for different subtypes of the κ -opioid receptor is available (Zukin et al. 1988; Clark et al. 1989; Rothman et al. 1989, 1992, 1993; Wollemann et al. 1993). Simonin et al. (1995) described cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system of the δ -opioid receptor in humans.

Salvinorin A – derived from *Salvia divinorum*, a hallucinogenic plant used by Mazatec Indians of Mexico for traditional spiritual ceremonies – is a highly selective κ -opioid receptor agonist with antinociceptive effects (Yan and Roth 2004; John et al. 2006; Rothman et al. 2006; Stewart et al. 2006; Vortherms and Roth 2006).

Endogenous ligands for κ receptors are **dynorphins**.

With the development of highly selective ligands it has become possible to label selectively each of the μ -, δ -, and κ -opioid binding sites.

The **μ -binding sites** are labeled with [3 H]-[Tyr-D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin (Kosterlitz and Paterson 1981), ¹²⁵I-FK 33–824 (Moyses et al. 1986), [3 H]-Tyr-Pro-MePhe-D-Pro-NH₂ (PL O17; Hawkins et al. 1987), or [3 H]-[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂] (CTOP); Hawkins et al. 1989), the **δ -binding sites** with [3 H]-[D-Pen²,D-Pen⁵]enkephalin (Akiyama et al. 1985; Cotton et al. 1985; Mosberg et al. 1987); [3 H]-D-Ser² (O-tert-butyl),Leu⁵]enkephalyl-Thr⁶ (Delay-Goyet et al. 1988) or [3 H]-[D-Pen²-pClPhe⁴,D-Pen⁵]enkephalin (Vaughan et al. 1989); [3 H]TIPP (Nevin et al. 1993), and the **κ -sites** with [3 H]-U-69593 (Lahti et al. 1985; Maguire et al. 1992), [3 H]-PD117302 (Clark et al. 1988), or [3 H]-CI-977 (Boyle et al. 1990) or [3 H]norBNI (Marki et al. 1995).

Cloning and molecular biology of opioid receptors has been reviewed (Reisine and Bell 1993).

Advances in research on non-peptide opioid receptor ligands were published by Kaczor and Matusiuk (2002).

Exploring the opioid system by gene knockout was described by Kieffer and Gaveriaux-Ruff (2002).

Oligomerization of opioid receptors and the generation of novel signaling units was discussed by Levac et al. (2003).

OTHER RECEPTORS

There is some evidence that other opioid receptors may exist, such as a β -endorphin-sensitive **ϵ receptor** (Wüster et al. 1981). The **ζ receptor** (Zagon et al. 1991) and a high affinity binding site referred to as the **λ site** (Grevel et al. 1985) may also be part of the opioid receptor system.

The existence of a **σ receptor** was first postulated by Martin et al. (1976) to account for the psychotomimetic effects of N-allylnormetazocine (SKF 10,047) in the chronic spinal dog. σ Binding sites were proposed to be identical to phencyclidine binding sites based on the finding that phencyclidine generalized to (+)-SKF 10,047 in drug discrimination tests. Further work led to the application of the **term σ to an unique class of non-opiate, non-phencyclidine sites** that may serve as receptors for an as yet unidentified neuro-modulator or neurotransmitter (Monnet et al. 1994). At least two subtypes of binding sites, σ_1 and σ_2 , are proposed (Bowen et al. 1989; Itzhak and Stein 1991; Karbon et al. 1991; Knight et al. 1991; Connick et al. 1992; Quirion et al. 1992; Leitner et al. 1994). Radioligands for σ receptors (Weber et al. 1986; de Costa et al. 1989) and for subtype σ_1 (Matsuno et al. 1996) and subtype σ_2 (Mach et al. 1999) were described. Pharmacological studies indicate a role of σ receptors not only in analgesia (Mach et al. 1999), but also in motor function (Walker et al. 1993), schizophrenia (Debonnel and de Montigny 1996; Guitard et al. 1998; Takahashi et al. 1999) and learning and memory (Maurice et al. 1999).

The heterogeneity of opioid receptors has been studied in **isolated tissue preparations** in which neurotransmission is sensitive to inhibition by opioids. The relative potencies of opioid agonists are assessed by their ability to inhibit the electrically evoked contractions of isolated tissue preparations from five different species: the contractions of the mouse **vas deferens** are inhibited by μ -, δ -, and κ -agonists (Maguire et al. 1992), those of the **guinea-pig myenteric plexus-longitudinal muscle** preparation by μ - and κ -agonists (Berzetei-Gurske 1992), those of the **rabbit vas deferens** by κ -agonists, and those of the **hamster vas deferens** by δ -agonists (Sheehan et al. 1986). The contractions of the **rat vas deferens** are inhibited mainly, but not exclusively, by δ -agonists. The actions of β -endorphin in the rat vas deferens are mediated by a further type of opioid receptors, termed **ϵ -receptor**

(Wüster et al. 1981; Corbett et al. 1992; Smith and Leslie 1992).

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H.1.1.2

3 H-Naloxone Binding Assay

PURPOSE AND RATIONALE

A good correlation between the *in vivo* pharmacological potency of opiate agonists and antagonists with their ability to displace radiolabeled naloxone has been reported. The later discovery that Na^+ (100 mM) enhances the binding of antagonists and reduces the binding of agonists has led to the development of an assay which is used to classify compounds as opiate agonists, mixed agonist-antagonists and antagonists by determining the IC_{50} values for ^3H -Naloxone in the presence or absence of Na^+ .

PROCEDURE

Reagents

[N-allyl-2,3- ^3H] Naloxone (38–58 Ci/mmol) is obtained from New England Nuclear.

For IC_{50} determinations ^3H -naloxone is made up to a concentration of 100 nM and 50 μl is added to each tube yielding a final concentration 5 nM in the assay.

Levorphanol tartrate is obtained from Hoffmann LaRoche. A stock solution of 1 mM levorphanol is made up in distilled water. This stock is diluted 1:200 in distilled water and 20 μl is added to 3 tubes to determine stereospecific binding yielding a final concentration of 0.1 μM in the assay.

Dextrophan tartrate is obtained from Hoffmann LaRoche. A stock solution of 1 mM dextrophan is made up in distilled water. This stock is diluted 1:200 in distilled water and 20 μl is added to the tubes containing the various concentrations of test drug and the tubes for total binding.

Test compounds: For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay

ranges from 10^{-5} to 10^{-8} M. At least 7 concentrations are used for each assay. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated and their brains rapidly removed. Whole brains minus cerebella are weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer with a Tekmar tissue homogenizer. The homogenate is centrifuged at 40,000 g for 15 min, the supernatant is decanted and the pellet resuspended in fresh buffer and recentrifuged at 40,000 g. The final pellet is resuspended in the original volume of fresh 0.05 M Tris buffer. This yields a tissue concentration in the assay of 10 mg/ml.

Assay

- 310 μ l H₂O
- 20 μ l 5 μ M dextrorphan (total binding) or
5 μ M levorphanol (non-specific binding)
- 50 μ l 2 M NaCl or H₂O
- 50 μ l 0.5 M Tris buffer, pH 7.7
- 20 μ l drug or vehicle
- 50 μ l ³H-naloxone
- 500 μ l tissue suspension.

The tubes are incubated for 30 min at 37°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with ice-cold 0.05 M Tris buffer, pH 7.7. The filters are then counted in 10 ml of Liquiscint liquid scintillation cocktail. Stereospecific binding is defined as the difference between binding in the presence of 0.1 μ M dextrorphan and 0.1 μ M levorphanol. Specific binding is roughly 1% of the total added ligand and 50% of the total bound in the absence of Na⁺ and 2% of the total added ligand and 65% of the total bound ligand in the presence of Na⁺ (100 mM). The increase in binding is due to an increase in specific binding.

EVALUATION

Data are converted into % stereospecific ³H-naloxone binding displaced by the test drug. *IC*₅₀ values are determined from computer-derived log-probit analysis. The sodium shift is calculated from *IC*₅₀ values with and without NaCl. High sodium shifts are found with agonists, low values with antagonists and medium values with mixed agonists-antagonists.

Data can be analyzed using a computer program as described by McPherson (1985).

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H.1.1.3

³H-Dihydromorphine Binding to μ Opiate Receptors in Rat Brain

PURPOSE AND RATIONALE

μ Receptors are considered to mediate the supraspinal activity of opioids. ³H-Dihydromorphine (³H-DHM) exhibits some selectivity for the μ receptor, a high affinity opiate binding site. The test is used to detect compounds that inhibit binding of ³H-DHM in a synaptic membrane preparation obtained from rat brain.

PROCEDURE

Reagents

[1,7,8-³H]Dihydromorphine (³H-DHM) (specific activity 69 Ci/mmol) is obtained from Amersham.

For *IC*₅₀ determinations a 20 nM stock solution is made up. Fifty μ l are added to each test tube to yield a final concentration of 0.5 nM in the 2 ml assay.

Levallorphan tartrate is used for the determination of nonspecific binding. A 0.1 mM stock solution is prepared in deionized water. Twenty μ l added to each of 3 tubes yields a final concentration of 0.1 μ M in the 2 ml assay.

A 1 mM stock solution is made up of the test compounds in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-6} to 10^{-9} M. At least 7 concentrations are used for each assay.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebella are removed, weighed and homogenized in 30 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 g for 15 min, the supernatant is decanted and the pellet resuspended in the same volume of buffer. This homogenate is then incubated for 30 min at 37°C to remove the endogenous opiate peptides and centrifuged again as before. The final pellet is resuspended in 50 volumes of 0.05 M Tris buffer, pH 7.7.

Assay

1850 µl tissue suspension
 80 µl distilled water
 20 µl vehicle, or levallorphan, or appropriate concentration of drug
 50 µl [³H]DHM.

Tubes are incubated for 30 min at 25°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed twice with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 0.1 mM levallorphan. *IC*₅₀ values are calculated from the percent specific binding at each drug concentration.

The *K*_D value for [³H]DHM binding was found to be 0.38 nM by Scatchard analysis of a receptor saturation experiment. The *K*_i value may be calculated from the *IC*₅₀ by the Cheng–Prusoff equation:

$$K_i = IC_{50} / (1 + L/K_D)$$

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H.1.1.4**³H-Bremazocine Binding to κ Opiate Receptors in Guinea Pig Cerebellum****PURPOSE AND RATIONALE**

κ Receptors are thought to be involved in the analgesic activity of opiates mainly within the spinal cord, whereas μ receptors are predominately located at supraspinal sites. The pharmacological effects of κ agonists differ from the μ agonists in various analgesic tests, effects on diuresis, sensitivity to naloxone and propensity to cause respiratory depression. κ Agonists may induce water diuresis (Salas et al. 1992). The receptor subtype selectivity can be determined by testing the affinity of new compounds for the κ opiate receptor and comparing these results with the data from the μ receptor assay.

Although the benzomorphanes, such as ethylketocyclazocine and bremazocine, are potent κ agonists, they are not selective for this receptor subtype. To demonstrate specific binding of these ligands to κ receptors, the assay must be done in a tissue where the κ subtype predominates, such as the guinea pig cerebellum. Moreover, binding to μ and δ receptors is prevented by inclusion of the peptide DAGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) to mask the μ receptor and of

[D-Pen^{2,5}]-enkephalin (Tyr-D-Pen-Gly-Phe-D-Pen) to mask the δ receptor.

PROCEDURE

Reagents

Bremazocine(-)-[9-³H] (specific activity 21–28 Ci/mmol), is obtained from, New England Nuclear. For IC_{50} determinations a 24 nM stock solution is made up. Fifty μ l are added to each tube to yield a final concentration of 0.6 nM in the 2 ml assay.

U50,488H is made up to a 500 μ M stock solution in deionized water. Twenty ml are added to each of the 3 tubes for determination of unspecific binding yielding a final concentration of 5.0 μ M in the 2 ml assay.

Opiate peptides of the μ - and δ -type are included in the assay to prevent binding of the radioligand and the test drug to these receptors. DAGO and [D-Pen^{2,5}]-enkephalin are obtained from Peninsula Laboratories. Concentrated stock solutions of 10^{-3} M are made up in deionized water and further diluted to 10^{-5} M. Twenty μ l of this solution are added to each tube to result in a final concentration of 100 nM of each in the 2 ml assay.

For the assays a 1 mM stock solution of test compounds is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. At least 7 concentrations are used for each assay.

Tissue Preparation

Male guinea pigs are sacrificed and cerebella are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris-buffer, pH 7.4. The homogenate is centrifuged at 48,000 g for 10 min, the supernatant decanted and the pellet resuspended in 20 volumes of buffer. This homogenate is then incubated for 45 min at 37°C to remove endogenous opiate peptides and centrifuged again as before. This pellet is resuspended in 200 volumes of 0.05 M Tris buffer, pH 7.4.

Assay

| | |
|--------------|------------------------------------|
| 1850 μ l | tissue suspension |
| 60 μ l | distilled water |
| 20 μ l | peptide solution |
| 20 μ l | vehicle, or U50,488H, or test drug |
| 50 μ l | [³ H]bremazocine |

Tubes are incubated for 40 min at 25°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of

0.05 M Tris buffer. The filters are then placed in scintillation vials with 10 ml Liquiscint scintillation cocktail and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 5.0 μ M U50,488H. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The K_D value for [³H]bremazocine binding was found to be 0.14 nM by Scatchard analysis of a receptor saturation experiment. The K_i value may be calculated from IC_{50} by the Cheng–Prusoff equation:

$$K_i = IC_{50}/1 + L/K_D .$$

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H.1.1.5

Inhibition of Enkephalinase

PURPOSE AND RATIONALE

Since the discovery of brain peptides with pharmacological properties similar to morphine (Hughes 1975), the metabolic breakdown of enkephalins has been studied (Malfroy et al. 1978; Llorens and Schwartz 1981; Mumford et al. 1981; Malfroy and Schwartz 1982; Roques 1982; Schwartz 1983). Roques BP et al. (1980), Costentin et al. (1986) found that the enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. A highly sensitive fluorometric assay for “enkephalinase”, a neutral metalloendopeptidase that releases tyrosine-glycine-glycine from enkephalins has been developed by Florentin et al. (1984). A fluorogenic peptide, dansyl-D-Ala-Gly-Phe(*p*NO₂)-Gly (DAGNPG) was synthesized as a selective substrate for the neutral metalloendopeptidase involved in enkephalin metabolism. This enzyme, designated “enkephalinase” cleaves the Gly-Phe(*p*NO₂) peptide bond of DAGNPG leading to a fluorescence increase related to the disappearance of intramolecular quenching of the dansyl fluorescence by the nitrophenyl residue.

Enkephalinase induces inactivation of atrial natriuretic factor (ANF). The protection of endogenous ANF against inactivation may result in therapeutic applications (Schwartz et al. 1990).

PROCEDURE

Fresh rat kidney is homogenized in 10 vol of cold 0.05 M Tris-HCl buffer, pH 7.4, using a Polytron homogenizer. The homogenate is centrifuged for 5 min at 1000 *g*. The pellet is discarded and the supernatant centrifuged at 60,000 *g* for 60 min. The resulting pellet is resuspended in 50 mM Tris-HCl buffer, pH 7.4, and used as the enzyme source.

Standard assays for “enkephalinase” activity using DAGNPG are carried out at 37°C in hemolysis tubes. A 0.1-ml amount of 50 mM Tris-HCl

buffer, pH 7.4, containing 50 μ M DAGNPG is preincubated 15 min at 37°C. The reaction is initiated by addition of 50 μ l of the enzyme preparation together with 0.5 μ M Captopril. The tubes are incubated for 30 min in a water bath with constant shaking. The enzymatic reaction is stopped by boiling at 100°C for 5 min. The samples are then diluted with 1.35 ml of Tris HCl buffer and centrifuged at 500 *g* for 30 min. An aliquot of 1 ml of the supernatant is transferred to thermostated cells of a spectrofluorometer. Readings are performed at 562 nm with an excitation wavelength of 342 nm. A calibration curve is prepared by adding increasing concentrations of DNS-D-Ala-Gly and decreasing concentrations of the substrate in Tris-HCl buffer containing the denaturated enzymatic preparation. For the assay of “enkephalinase” inhibition, the test compound or the standard thiorphan = [(R-,S-)-3-mercapto-2-benzylpropanoyl]glycine is added in various concentrations.

EVALUATION

The inhibitory potencies of test compounds are compared with the standard.

MODIFICATIONS OF THE METHOD

Ksander et al. (1989) incubated synaptic membranes from rat striatum with ³H-Tyr-Leu-enkephalin for 15 min at 30°C, pH 6.5, in the presence of 10⁻⁶ M bestatin. The reaction was stopped by the addition of 30% acetic acid and the reaction product ³H-Tyr-Gly-Gly was separated from unreacted ³H-Tyr-Leu-enkephalin on a Porapak Q column followed by a Cu²⁺ chelex column. The ³H-Tyr-Gly-Gly was counted by liquid scintillation.

The antinociceptive effects of intrathecally administered SCH32615, an enkephalinase inhibitor were studied in the rat by Oshita et al. (1990).

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H.1.1.6

Nociceptin

H.1.1.6.1

General Considerations on Nociceptin

PURPOSE AND RATIONALE

A heptadecapeptide (nociceptin or orphanin FQ) has been isolated as endogenous agonist of the **opioid receptor-like ORL₁ receptor** (Reinscheid et al. 1995; Meunier et al. 1995; Barlocco et al. 2000; Mollereau and Mouledous 2000; Mogil and Pasternak 2001; Witta et al. 2004) which shows high structural homology with opioid peptides, especially dynorphin A (Calò et al. 2000). Nociceptin activates a specific receptor, which has been cloned in man and animals and has been shown to be structurally similar to opioid receptors (Mollereau et al. 1994; Calò et al. 2000; Hawkinson et al. 2000). At the cellular level, the nociceptin receptor has been shown to act through the same mechanisms as classical opioid receptors, namely the inhibition of adenylyl cyclase, the activation of potassium channels and inhibition of calcium

channels (Connor et al. 1996a, b). *In vitro* and *in vivo* studies have demonstrated that nociceptin mediates a variety of biological actions (Civelli et al. 1998; Darland et al. 1998). Nociceptin induces analgesia when administered intrathecally (Stanfa et al. 1996; Xu et al. 1996), while it causes hyperalgesia and reversal of opioid induced analgesia when given intracerebroventricularly; nociceptin stimulates food intake (Polidori et al. 2000) and produces anxiolysis. Depending on the dose, nociceptin stimulates or inhibits locomotor activity. Nociceptin inhibits long-term potentiation, memory processes, induces bradycardia, hypotension and diuresis. In addition, nociceptin inhibits neurotransmitter release both at central and peripheral sites. Intracavernosal injection of nociceptin induces a potent and relatively long-lasting erectile response in the cat (Champion et al. 1997, 1998). Intrathecal injection of nociceptin elicits scratching, licking and biting in mice (Sakurada et al. 1999, 2000). Synthetic agonists and antagonists of the nociceptin receptor have been reported (Guerrini et al. 1998; Salvadori et al. 1999; Calò et al. 2000; Hashimoto et al. 2000; Meunier 2000; Ozaki et al. 2000).

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H.1.1.6.2

Receptor Binding of Nociceptin

PURPOSE AND RATIONALE

The nociceptin receptor has been termed by different groups of investigators as ORL1, LC132, ROR (see Meunier 1997). Based on the structural and transductional similarities between receptors for nociceptin and those for opioids, Hamon (1998) proposed to include the nociceptin receptor in the opioid receptor family with the name OP₄.

Varani et al. (1999) tested synthetic nociceptin analogs for their displacement at the nociceptin- and at classical opioid receptors. The displacement of [³H]N₂NCNH₂ ([³H]nociceptin amide, ORL1 site), and of the selective opioid receptor ligands [³H]DAMGO (μ site), [³H]deltorphin II (δ site), and [³H]U69593 (κ site) was studied.

Wisner et al. (2006) described human opiorphin, a natural antinociceptive modulator of opioid-dependent pathways inhibiting enkephalin-inactivating zinc ectopeptidases.

PROCEDURE

Membrane Preparation

Guinea pigs are decapitated and the whole brain (without cerebellum) rapidly removed. The tissue is disrupted in a Polytron homogenizer (setting 5) in 50 mM Tris HCl, pH 7.4, to prepare membranes for the classic opioid receptor studies. The homogenate is centrifuged at 4000 g for 10 min and the pellet is resuspended with a Polytron PTA 10 probe (setting 5) in the same ice-cold buffer. To study the binding to ORL1 receptor, the tissue is homogenized in 50 mM Tris HCl, 2 mM EDTA and 100 μ M phenylmethylsulphonylfluoride HCl (PMSF) at pH 7.4. The suspension is centrifuged at 40,000 g for 10 min and the pellet is resuspended in the same buffer. After 30 min of incubation at 37°C the membranes are centrifuged at 40,000 g for 10 min and the pellets are stored at –70°C. The protein concentration is determined with bovine albumin as standard.

Binding Assays

Classic opioid receptors, μ , δ and κ , are studied according to Bhargava and Zhao (1996). Saturation binding experiment are carried out using 8–10 different concentrations of [³H]DAMGO ranging from 0.15 nM to 15 nM, [³H]deltorphin II from 0.1 nM to 10 nM, and [³H]U69593 from 0.15 nM to 15 nM, respectively. Inhibition experiments are carried out in duplicate in a final volume of 250 μ l in test tubes containing ei-

ther 1.5 nM [³H]DAMGO or 1.0 nM [³H]deltorphin II or 1.5 nM [³H]U69593, 50 mM Tris HCl at pH 7.4, guinea pig brain membranes (150–200 μg of protein/assay) and at least 8–10 different concentrations of the ligands under study. Binding assays to the ORL1 receptor are carried out according to Varani et al. (1998). In saturation studies, membranes are incubated with 8–10 different concentrations of [³H]N₂CNH₂ ([³H]nociceptin amide) ranging from 0.1 mM to 10 mM. Inhibition experiments are carried out in duplicate in a final volume of 250 μl in test tubes containing 1 mM [³H]N₂CNH₂, 50 mM Tris HCl, 2 mM EDTA, 100 μM phenylmethylsulphonyl fluoride HCl (PMSF) at pH 7.4, guinea pig membranes, and at least 8–10 different concentration of the compound under examination. The incubation time is 1 h for [³H]DAMGO and [³H]U69593 and 2 h for [³H]deltorphin II and [³H]N₂CNH₂. Nonspecific binding is defined as the binding measured in the presence of 100 μM bremazocine for classic opioid receptors and 10 μM N₂CNH₂ for ORL1 receptors.

Bound and free radioactivity are separated by filtering the assay mixture through Whatman GF/B glass-fibre filters, previously treated with PEI 0.1%; the incubation mixture is diluted with 3 ml of ice-cold incubation buffer, rapidly filtered by vacuum, and the filter washed three times with 3 ml of incubation buffer. The filter-bound radioactivity is measured in a Beckman LS-1800 Spectrometer.

EVALUATION

The inhibitory binding constant (K_i) values are calculated from the IC_{50} values according to the Cheng and Prusoff equation. The weighted non-linear last-squares curved fitting program LIGAND (Munson and Rodbard 1980) is used for computer analysis of saturation and inhibition experiments.

MODIFICATIONS OF THE METHOD

Ardati et al. (1997) developed two radioligands for the orphanin FQ receptor: a tritiated OFQ peptide ([³H]orphanin FQ) and radioiodinated form in which Leu¹⁴ is substituted by tyrosine (¹²⁵I-Tyr¹⁴-orphanin FQ). Both exhibit virtually identical characteristics.

Seki et al. (1999) analyzed the pharmacological properties of κ -opioid receptor-selective agonist TRK-820 using Chinese hamster ovary cells expressing cloned rat μ -, δ - and κ -opioid receptors and human nociceptin receptor.

Mouledous et al. (2000) reported a site-directed mutagenesis study of the ORL1 receptor transmembrane-binding domain.

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H.1.1.6.3

Bioassays for Nociceptin

PURPOSE AND RATIONALE

Nociceptin receptors in the periphery can be characterized by studies in isolated organs (Guerrini et al. 1998; Bigoni et al. 1999): the guinea pig ileum according to Paton (1957) (see J.4.3.1), the mouse vas deferens according to Hughes et al. (1975), the rabbit vas deferens according to Oka et al. (1980) (see A.1.2.3), the guinea pig renal pelvis (Giuliani and Maggi 1996) (see C.4.2.1).

PROCEDURE

Tissues are taken from male Swiss mice (25–30 g), guinea pigs (300–350 g) Sprague Dawley rats (300–350 g) and New Zealand albino rabbits (1.5–1.8 kg). They are suspended in 10 ml organ baths containing Krebs solution oxygenated with 95% O₂ and 5% CO₂. The temperature is set at 33°C for the mouse vas deferens and at 37°C for the other tissues. A resting tension of 0.3 g is applied to the mouse deferens, 1 g to the guinea pig ileum, rats vas deferens, and rabbit vas deferens and 0.15 g to the guinea pig renal pelvis. For experiments at the mouse vas deferens a Mg²⁺-free Krebs solution is used and for rat vas deferens experiments a Krebs solution containing 1.8 mM CaCl₂. Guinea pig renal pelvis experiments are performed in the presence of indomethacin (3 μM).

The mouse vas deferens, guinea pig ileum, rat vas deferens, and rabbit vas deferens are continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.1 Hz frequency. The electrically evoked contractions are measured isotonicity with a strain gauge transducer and recorded on a multichannel chart recorder. After an equilibration period of about 60 min the contractions induced by electrical field stimulation are stable; at this time, cumulative concentration response curves to nociceptin or opioid peptides are performed (0.5 log unit steps).

The guinea pig renal pelvis is stimulated through two platinum ring electrodes with 100 V square wave pulses of 1 ms duration at a frequency of 5 Hz for 10 s. The spontaneous activity and the positive inotropic responses to electrical field stimulation are measured by an isotonic transducer and recorded by a two channel recorder. The experiments are started following a 60 min equilibration period. Four electrical field stimulation are performed with each tissue at 30 min intervals. Agonists are added to the bath 5 min, and antagonists 15 min before the next stimulus. The contractile responses to electrical field stimulation are expressed as % increment the spontaneous activity of the tissue; the biological effects of the application of agonists or antagonists are expressed as % inhibition of electrical field stimulation-induced contraction.

EVALUATION

Data are expressed as means ± SEM of *n* experiments and statistically analyzed with Student two-tailed *t*-test of one way ANOVA plus Dunnett test. The agonist potencies are given as pE₅₀, which is the

negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given preparation. Antagonist potencies are expressed in terms of pA₂, which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response.

MODIFICATIONS OF THE METHOD

Rizzi et al. (1999) studied nociceptin and nociceptin analogs in the isolated mouse colon.

Bigoni et al. (1999) used nociceptin, a series of nociceptin fragments, naloxone as well as [Phe¹Ψ (CH₂-NH)Gly²]nociceptin(1–13)NH₂ and [Nphe¹]nociceptin(1–13)NH₂ to characterize nociceptin receptors in peripheral organs, such as mouse and rat vas deferens (noradrenergic nerve terminals), in the guinea pig ileum (cholinergic nerves) and renal pelvis (sensory nerves) and *in vivo* by measuring the blood pressure and heart rate in anesthetized rats.

Menzies et al. (1999) described the agonist effects of nociceptin and [Phe¹Ψ (CH₂-NH)Gly²]nociceptin(1–13)NH₂ in the mouse and rat colon and in the mouse vas deferens.

Kolesnikov and Pasternak (1999) found an ED₅₀ of 16.3 μg after peripheral administration of nociceptin in the tail flick test in mice.

Bortorelli et al. (1999) found anti-opioid effects of nociceptin and the ORL1 ligand [Phe¹Ψ (CH₂-NH)Gly²]nociceptin(1–13)NH₂ in the Freund's adjuvant-induced arthritic rat model of chronic pain.

Yamamoto and Sakashita (1999) studied the effect of nocistatin, a 17 amino acid peptide which is processed from prepronociceptin and its interaction with nociceptin in the rat formalin test.

Hashiba et al. (2003) measured the effects of nociceptin/orphanin FQ receptor ligands on blood pressure, heart rate, and plasma catecholamine concentrations in guinea pigs.

Using the forced swimming test and the tail suspension test in rats and mice, Gavioli et al. (2004) demonstrated antidepressant-like effects of the nociceptin/orphanin FQ receptor antagonist UFP-101.

Varty et al. (2005) characterized a nociceptin receptor (ORL-1) agonist in tests of anxiety across three species: rat, guinea pig, and mouse.

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H.1.1.7

Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase-Activating Peptide (PACAP)

PURPOSE AND RATIONALE

Several peptides are considered to play a role in the altered transmission of sensory information in neuropathic conditions, such as neuropathic pain arising from trauma or compression injury of peripheral nerves (Zhang et al. 1998).

Vasoactive intestinal polypeptide (VIP), isolated by Nakajima et al. (1970), is a neuropeptide of 28 amino acids with widespread distribution in both the central and peripheral nervous system (Fahrenkrug 1979; Gafvelin 1990).

Together with the structurally related pituitary adenylate cyclase-activating peptide (PACAP) this peptide is considered to play an important role in the somatosensory processing of pain (Dickinson and Fleetwood-Walker 1999). PACAP-38 (a 38 amino acid polypeptide) and the C-terminally truncated form PACAP-27 share 68% amino acid homology at their N-terminal domain with VIP. A shorter peptide with 27 amino acids, named as PACAP27 was described by Miyata et al. (1990). These peptides are members of a superfamily of hormones that includes glucagon, glucagon-like peptide, secretin and growth hormone releasing factor.

Three G-protein-coupled receptors are described: the VPAC₁ receptor, originally described as the VIP receptor and subsequently designated as VIP₁ receptor; the VPAC₂ receptor, previously designated VIP₂; and the PAC₁ receptor, previously known as PACAP type I receptor (Buscail et al. 1990; Guijarro et al. 1991; Felley et al. 1992; Calvo et al. 1994; Van Rampelbergh et al. 1996; Harmar et al. 1998; Robberecht et al. 1999).

Many peripheral activities of VIP/PACAP are described, such as stimulation of pancreatic secretion (Onaga et al. 1997; Ito et al. 1998; Soo Tek Lee et al. 1998); stimulation of duodenal bicarbonate secretion (Takeuchi et al. 1998); relaxation of smooth muscle cells in the intestinal tract, e.g., gall bladder (Pang and Kline 1998), cecal circular smooth muscle (Motomura et al. 1998), internal anal sphincter (Rattan and Chakder 1997); on duodenal motility (Onaga et al. 1998); enhancement of insulin secretion (Yada et al. 1997; Filipsson et al. 1998); bronchodilation (Linden et al. 1998; Shigyo et al. 1998; Okazawa et al. 1998). Centrally administered PACAP showed an anorectic effect (Mizuno et al. 1998).

Agonists (Gourlet et al. 1997a, b) and antagonists (Gozes et al. 1991; Gourlet et al. 1997c) for VIP were described. Further studies are aimed to development of drugs for neuropathic analgesia, ultimately of non-peptide nature, using VPAC₁, VPAC₂, and PAC₁, receptors as drug targets (Dickinson and Fleetwood-Walker 1999).

PROCEDURE

CHO cell lines expressing the rat VIP₁ receptor (Ciccarelli et al. 1994), the human VIP₂ receptor (Sreedharan et al. 1993), the rat PACAP I receptor (Ciccarelli et al. 1995), and the rat secretin receptor (Ishihara et al. 1991) are used.

Transfected CHO cells are harvested with a rubber policeman and pelleted by low speed centrifugation. The supernatant is discarded and the cell lysed in mM NaHCO₃ solution and immediate freezing in liquid nitrogen. After thawing, the lysate is first centrifuged at 4°C for 10 min at 400 g and the supernatant is further centrifuged at 20,000 g for 10 min. The pellet, re-suspended in 1 mM NaHCO₃ is used immediately as a crude membrane fraction.

Binding is performed using [¹²⁵I]VIP (specific radioactivity of 0.5 Ci/nmol), [¹²⁵I]Tyr²⁵ secretin (specific radioactivity of 1.0 Ci/nmol) and [¹²⁵I]-Ac-His¹]PACAP-27 (specific radioactivity of 0.7 Ci/nmol) as tracers. In all cases, non-specific binding is defined as the residual binding in the presence of 1 μM of the unlabeled peptide corresponding to the tracer. Binding is performed at 37°C in a 20 mM Tris-maleate, 2 mM MgCl₂, 0.1 mg/ml bacitracin, 1% bovine serum albumin (pH 7.4) buffer. Bound radioactivity is separated from free by filtration through glass-fibre GF/C filters presoaked for 24 h in 0.1% polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) sodium phosphate buffer containing 1% bovine serum albumin.

EVALUATION

The IC₅₀ values (in mM) for each peptide on each receptor are calculated from complete dose-effect curves performed on three different membrane preparations using the LIGAND program.

MODIFICATIONS OF THE METHOD

Schmidt et al. (1993) studied the binding of PAPAC, VIP and analogues of VIP and PAPAC in rat AR 4-2J pancreatic carcinoma cells and isolated pancreatic acini to the PAPAC-1 receptor, abundantly expressed in AR 4-2J pancreatic carcinoma cells, and to the VIP/PAPAC-2 receptor. Simultaneously, biological effects (lipase secretion and cAMP production) in pan-

creatic acini were determined. PAPAC was regarded as a potent ligand for both receptor types and as a potent VIP-like secretagogue.

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H.1.1.8

Cannabinoid Activity

H.1.1.8.1

General Considerations on Cannabinoids

In the centuries since hashish and marijuana (*Cannabis sativa*) were used as psychoactive drugs the most significant discoveries in regard to the mechanism of action were made with the isolation of (–)-trans- Δ^9 -tetrahydrocannabinol (Δ^9 -THC) as the principal active ingredient (Mechoulam et al. 1970), the characterization and localization of the cannabinoid receptor in the brain (Devane et al. 1988), the cloning of its gene (Matsuda et al. 1990), and the identification of an endogenous ligand (Devane et al. 1992). Most cannabinoid effects occur receptor mediated in the CNS (Martin 1986; Herkenham et al. 1990; Porter and Felder 2001; Howlett et al. 2002). The recognized CNS responses to cannabinoids include alterations in cognition and memory, euphoria and sedation (Howlett 1995). Ranganathan and D'Souza (2006) reviewed the acute effects of cannabinoids on memory in humans.

Cannabinoids have been shown to produce analgesia without the respiratory problems associated with opioid analgesics (Buxbaum 1972; Martin 1985; Dewey 1986; Razdan 1986; Compton et al. 1992; Meng et al. 1998; Strangman et al. 1998) which may be of value for therapeutic applications (Hollister 1986; Izzo et al. 2000a; Pertwee 2000). Simultaneous administration of cannabinoid receptor agonists and μ - or κ -receptor agonists indicate a cannabinoid-opioid interaction in anti-nociception (Manzanares et al. 2000). Baker et al. (1990, 2000) described experimental allergic encephalomyelitis with relapsing-remitting episodes, spasticity and tremor similar to multiple scler-

rosis in human beings in Biozzi AB/H mice. These symptoms could be antagonized by cannabinoids.

A multiple-evaluation paradigm of *in vivo* mouse assays is employed to test for cannabimimetic effects. This paradigm includes assays for reduction in spontaneous activity, and the production of hypothermia, catalepsy, and antinociception measured by tail-flick assay (Compton et al. 1992; Welch et al. 1998). The behavioral effects of Δ^9 -THC and related cannabinoids in mice have been termed the “popcorn” effect. That is, groups of mice are in a sedated state with little or no movement until a stimulus causes one mouse to jump (hyper-reflexia). This animal falls on another mouse which in turn jumps so that this repeated hyper-reflexic jumping looks like corn popping in a machine. Subsequently, all mice will be sedated until another stimulus reinitiates the process (Dewey 1986). Like the opioids, cannabinoids inhibit electrically evoked contractions of the mouse vas deferens and the guinea pig ileum, but unlike the opioids, these effects are not antagonized by naloxone (Pertwee et al. 1992; Hillard et al. 1999).

In addition to the effects in the CNS (Chaperon and Thiebot 1999), peripheral effects of cannabinoids are known (Lynn and Herkenham 1994) including actions on the **endocrine system** (Patra and Wadsworth 1990, (Block et al. 1991; Wenger et al. 2000), on the **digestive tract** (Rosell and Agurell 1975; Izzo et al. 1990a, b, 2000; Coutts et al. 2000; Massa et al. 2005), on **ingestive behavior** (Giuliani et al. 2000), on the **pulmonary and cardiovascular system** (Stengel et al. 1998; White and Hiley 1998; Niederhoffer and Szabo 1999; Liu et al. 2000; Niederhoffer et al. 2003), and on **immune modulation** (Kaminski et al. 1992; Lynn and Herkenham 1994; Achiron et al. 2000).

An endogenous cannabinoid was isolated from porcine brain by Devane et al. (1992) and found to be an unsaturated fatty acid ethanolamide, arachidonylethanolamide, also called **anandamide**, which activates CB1 receptors (Devane et al. 1992; Hillard and Jarrahian 2005) and produces similar effects as Δ^9 -tetrahydrocannabinol including anti-nociception, hypothermia, hypomotility and catalepsy in mice (Smith et al. 1994). The brain enzyme hydrolyzing and synthesizing anandamide has been characterized by Ueda et al. (1995). The human brain fatty-acid amide hydrolase was characterized by Maccarrone et al. (1998) as a single protein, which hydrolyses anandamide to arachidonate and ethanolamine.

Similar effects are produced by other polyunsaturated N-acetylethanolamines, such as N-palmitoyl-ethanolamine, which activates the CB-2-like receptor

subtype (Hanu et al. 1993; Facci et al. 1995). Both endogenous cannabinoids (called endocannabinoids) derive from cleavage of a precursor phospholipid, N-acylphosphatidylethanolamine, catalyzed by Ca^{2+} -activated D-type phosphodiesterase activity (Cadas et al. 1996). 2-Arachidonylglycerol was described as a further endogenous ligand for cannabinoid receptors (Ameri and Simmet 2000; Sigiura et al. 2000)

Numerous synthetic analogs and cannabimimetic compounds have been evaluated as agonists and antagonists by *in vitro* and *in vivo* pharmacological methods (Martin et al. 1991; D’Ambra et al. 1992; Melvin et al. 1993; Barth and Rinaldi-Carmona 1999; Hillard et al. 1999; Palmer et al. 2000; Piomelli et al. 2000; De Petrocellis et al. 2004; Costa et al. 2005; Griebel et al. 2005; Lange et al. 2005; Makriyannis et al. 2005; Pertwee 2006).

Costa et al. (2004) described oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw.

For studies on the effects of cannabinoids and cannabinoid antagonists on digestive system and obesity see L.3.1.3.

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H.1.1.8.2

Receptor Binding of Cannabinoids

PURPOSE AND RATIONALE

After the discovery of cannabinoid receptors in brain (Howlett et al. 1988; Devane et al. 1988; Pertwee 1993), two cannabinoid receptor subtypes were identified: CB1 and CB2. Cannabinoid receptors and were reviewed by Felder and Glass (1998), Pertwee (1999, 2001).

CB1 has an amino acid sequence consistent with a tertiary structure typical of the seven transmembrane-spanning proteins that are coupled to G proteins (Gerard et al. 1990, 1991; Howlett et al. 1990; Matsuda et al. 1990). The CNS responses to cannabinoid compounds are apparently mediated exclusively by CB1, since CB2 transcripts could not be found in brain tissue. CB1 transduces signals in response to CNS active constituents of *Cannabis sativa*, as well as synthetic bicyclic and tricyclic analogs, aminoalkylindole, and eicosanoid cannabi-mimetic compounds. CB1 is coupled to G₁ to inhibit adenylate cyclase activity and to a pertussis-sensitive G protein to regulate Ca²⁺ currents. Zimmer et al. (1999) produced a mouse strain with a disrupted CB1 gene. These CB1 knockout mice had a significantly increased mortality rat and displayed reduced locomotor activity, increased ring catalepsy, and hypoalgesia in hot plate and formalin tests.

CB2, the second cannabinoid-binding seven-transmembrane spanning receptor, exhibits 68% identity to CB1 within the helical regions, and 44% identity throughout the total protein. The CB2 clone was derived from a human promyelocytic leukemia cell line HL60 cDNA library (Munro et al. 1993), also expressed in human leukocytes (Bouaboula et al. 1993). The gene for the rat CB2 receptor was cloned,

expressed, and its properties compared with those of mouse and human CB2 receptors (Griffin et al. 2000).

Receptor binding of cannabinoids in correlation to *in vivo* activities was described by Compton et al. (1993).

PROCEDURE

Membrane Preparation

Male Sprague Dawley rats weighing 150–200 g are decapitated and the brain rapidly removed. The cortex is dissected free using visual landmarks following reflection of cortical material from the midline and immersed in 30 ml of ice-cold centrifugation solution (320 mM sucrose, 2 mM Tris-EDTA, 5 mM MgCl₂). The process is repeated until the cortices of five rats are combined. The cortical material is homogenized with a Potter-Elvehjem glass-Teflon grinding system. The homogenate is centrifuged at 1600 *g* for 15 min, the supernatant saved and combined with the two subsequent supernatants obtained from washing and 1600 *g* centrifugation of the P₁ pellet. The combined supernatant fractions are centrifuged at 39,000 *g* for 15 min. The P₂ pellet is resuspended in 50 ml buffer (50 mM Tris-HCl, 2 mM Tris EDTA, 5 mM MgCl₂, pH 7.0), incubated for 10 min at 37°C, then centrifuged at 23,000 *g* for 10 min. The P₂ membrane is resuspended in 50 ml of buffer A, incubated again except at 30°C for 40 min, then centrifuged at 11,000 *g* for 15 min. The final wash-treated P₂ pellet is resuspended in assay buffer B (50 mM Tris-HCl, 1 mM Tris EDTA, 3 mM MgCl₂, pH 7.4) to a protein concentration of approximately 2 mg/ml. The membrane preparation is divided into 4 aliquots and quickly frozen in a bath solution of dry ice and 2-methylbutane and then stored at –80°C.

Binding Assay

Binding is initiated by the addition of 150 mg of P₂ membrane to test tubes containing [³H]CP-55,940 (79 Ci/mmol), a cannabinoid analog, (for displacement studies) and a sufficient quantity of buffer C (50 mM Tris-HCl, 1 mM Tris EDTA, 3 mM MgCl₂, 5 mg/ml BSA) to bring the total incubation volume to 1 ml. The concentration of [³H]CP-55,940 in displacement studies is 400 pM, whereas that in saturation studies varies from 25 to 2500 pM. Nonspecific binding is determined by the addition of 1 mM unlabeled CP-55,940. The standard CP-55,940 and other cannabinoid analogs are prepared in suspension buffer C from a 1 mg/ml ethanolic stock without evaporation of the alcohol.

After incubation at 30°C for 1 h, binding is terminated by addition of 2 ml ice-cold buffer D (50 mM

Tris-HCl, 1 mg/ml BSA) and vacuum filtration through pretreated filters in a 12-well sampling manifold. Reaction vessels are washed once with 2 ml of ice-cold buffer D, and the filters washed twice with 4 ml of ice-cold buffer D. Filters are placed into 20-ml plastic scintillation vials with 1 ml of distilled water and 10 ml of Budget-Solve (RPI Corp., Mount Prospect, IL). After shaking for 1 h, the radioactivity present is determined by liquid scintillation photometry.

EVALUATION

The B_{\max} and K_d values obtained from Scatchard analysis are determined via a suitable computer program. Displacement IC_{50} values are determined by unweighted least squares linear regression of log concentration-percent displacement data and then converted to K_i values.

MODIFICATIONS OF THE METHOD

To further characterize neuronal cannabinoid receptors, Thomas et al. (1998) compared the ability of cannabinoid analogs to compete for receptor sites labeled either with [³H]SR141716A or [³H]CP-55940.

Herkenham et al. (1991) characterized and localized cannabinoid receptors in rat brain by a quantitative autoradiographic study.

Felder et al. (1995) compared the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors.

Ross et al. (1998) compared cannabinoid binding sites in guinea pig forebrain and small intestine.

Rinaldi-Camora et al. (1998) tested the affinity of an antagonist of the CB2 cannabinoid receptor for rat spleen and cloned human CB2 receptors.

Bilkei-Gorzo et al. (2005) described early age-related cognitive impairment in mice lacking cannabinoid CB1 receptors.

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- et al. 1997; Sterner and Szallasi 1999; Szallasi and Blumberg 1999; Caterina and Julius 2001; Piomelli 2001; Gunthorpe et al. 2002). Capsaicin was isolated by Thresh (1846). The chemical structure was determined by Nelson (1919). The analgesic use of capsaicin was reviewed by Lembeck (1987). Capsaicin excites a subset of primary sensory neurons with somata in the dorsal root ganglion or trigeminal ganglion. As a general rule, these vanilloid-sensitive neurons are peptidergic, small diameter (50 µm) neurons, giving rise to thin, unmyelinated C fibers. Among sensory neuropeptides, the tachykinin Substance P shows the best correlation with vanilloid sensitivity. Vanilloid-sensitive neurons transmit noxious information (usually perceived as itching or pain) to the CNS, whereas peripheral terminals rare sites of release of a variety of pro-inflammatory neuropeptides. Among irritant compounds acting on primary sensory neurons, capsaicin and related vanilloids are unique in that the initial stimulation by vanilloids is followed by a long lasting refractory state. Neurotoxicity has been observed when capsaicin as given to newborn rats (Jancsó et al. 1977; Nagy and van der Kooy 1983).
- Besides capsaicin, several natural vanilloid agonists were described (Jonassohn and Sterner 1997; Liu et al. 1997; Sterner and Szallasi 1999; Mendes et al. 2000). The irritant principle from *Euphorbia resinifera*, named resiniferatoxin, was isolated by Hergenhahn et al. (1975). In several assays, resiniferatoxin and its derivatives are several thousand-fold more potent than capsaicin (Szolcsanyi et al. 1990; Ács et al. 1995), which is explained by specific receptor binding (Szallasi and Blumberg 1990; Ács et al. 1994). Lee et al. (2001) described simplified resiniferatoxin derivatives as potent vanilloid receptor agonists with potent analgesic activity and reduced pungency.
- The high affinity of vanilloid receptors argues for the existence of endogenous vanilloids. Hwang et al. (2000), Piomelli (2001) reported a direct activation of capsaicin receptors by products of lipogenases. Pain-inducing substances, such as bradykinin, may activate phospholipase-linked receptors in sensory neurons, mobilizing arachidonic acid from phospholipids and generating 12-HPETE. This lipid second messenger interacts in turn with a cytosolic domain of the VR1 receptor channel, increasing its opening probability and causing the sensory neuron to become depolarized.
- The endogenous ligand of CB₁ cannabinoid receptors, anandamide, is also a full agonist at vanilloid VR1 receptors (Zygmunt et al. 1999; Maccarrone et al. 2000; Smart et al. 2000; DePetrocellis et al. 2001).

H.1.1.9

Vanilloid (Capsaicin) Activity

H.1.1.9.1

General Considerations on Vanilloids

PURPOSE AND RATIONALE

Several authors reviewed the recent development of capsaicin and vanilloid receptors (Holzer P 1991; Bíró

Premkumar and Ahern (2000) showed that activation of protein kinase C activates VR1 channel activity.

The first capsaicin or vanilloid receptor, termed VR1, was cloned by Caterina et al. (1997). Hayes et al. (2000) reported the cloning and functional expression of a human orthologue of rat vanilloid receptor 1. Pharmacological differences between the human and rat vanilloid receptor 1 were observed (McIntyre et al. 2001). VR1 functions as a molecular integrator of painful chemical and physical stimuli including capsaicin, noxious heat and low pH (Tominaga et al. 1998; Michael and Priestley 1999; Davis et al. 2000; Welch et al. 2000). In mice lacking the capsaicin receptor impaired nociception and pain sensation was observed (Caterina et al. 2000).

Vanilloid receptors are differently distributed in the central and peripheral nervous system (Szallasi 1995; Szallasi et al. 1995; Mezey et al. 2000; Ichikawa and Sugimoto 2001). Bíró et al. (1998) reported characterization of functional vanilloid receptors expressed by mast cells. Biological and electrophysiological data indicate heterogeneity within the vanilloid receptors. Caterina et al. (1999) described a capsaicin-receptor homologue, named vanilloid-receptor-like protein (VRL-1) with a high threshold for noxious heat. A novel human vanilloid receptor-like protein, named VRL-2 was identified and characterized by Delany et al. (2001).

Price et al. (2004) reported modulation of trigeminal sensory neuron activity by the dual cannabinoid-vanilloid agonists anandamide, *N*-arachidonoyl-dopamine and arachidonoyl-2-chloroethylamide.

Several vanilloid antagonists were described, such as capsazepine (Bevan et al. 1992; Walpole et al. 1994) or iodo-resiniferatoxin (Wahl et al. 2001).

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H.1.1.9.2

Vanilloid Receptor Binding

PURPOSE AND RATIONALE

Ács et al. (1994) described [³H]resiniferatoxin binding by the human vanilloid (capsaicin) receptor. Receptor types and species differences of the vanilloid receptor were described by Szallasi et al. (1994, 1996). The rat vanilloid receptor (rVR1) was cloned and stably expressed in HEK293 cells by Jerman et al. (2000). A detailed pharmacological characterization was conducted using the Ca²⁺-sensitive dye, Fluo3AM in a fluorimetric imaging plate reader (FLIPR). Ross et al. (2001) studied structure-activity relationship for the endogenous cannabinoid, anandamide, and certain of its analogues at vanilloid receptors in transfected CHO cells.

PROCEDURE

Cell Culture

Rat vanilloid receptor (rVR1) transfected CHO cells are maintained in MEM Alpha minus media containing 2 mM L-glutamine supplemented with 10% hyclone fetal bovine serum, 350 µg/ml G418 (Sigma-Aldrich), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells are maintained in 5% CO₂ at 37°C and passed twice a week using non-enzymatic cell dissociation solution. For the radioligand binding assay, cells are removed from flasks by scraping and then frozen as a pellet at –20°C for up to one month.

Radioligand Binding Experiments

Assays are performed in DMEM containing HEPES (25 mM) and BSA (0.25 mg/ml). Total assay volume is 500 μ l containing 20 μ g of cell membranes. Binding is initiated by addition of [³H]resiniferatoxin ([³H]-RTX). Assays are carried out at 37°C for 1 h, before termination by addition of ice-cold wash buffer (50 mM Tris-buffer, 1 mg/ml BSA, pH 7.4) and vacuum filtration using a 12-well sampling manifold (Brandell cell harvester) and Whatman GF/B filters that have been soaked in wash buffer at 4°C for at least 24 h. Each reaction is washed 9 times with a 1.5 ml aliquot of wash buffer. The filters are oven-dried and then placed in 5 ml scintillation fluid. Radioactivity is quantified by liquid scintillation spectrometry. Specific binding is determined in the presence of 1 μ M unlabelled RTX. Protein assays are performed using a Bio-Rad De Kit. Unlabelled compounds are added in a volume of 50 μ l after serial dilution using assay buffer from a 10 mM stock in ethanol or DMSO. [³H]-RTX is also added in a 50 μ l volume following dilution in assay buffer.

EVALUATION

The K_D value and B_{max} for [³H]-RTX and the concentration of competing ligands to produce 50% displacement of the radioligand (IC_{50}) from specific binding sites are calculated using GraphPad Prism (GraphPad Software, San Diego). Dissociation constant (K_i) values are calculated using the Cheng and Prussoff equation.

MODIFICATIONS OF THE METHOD

Wardle et al. (1997) used a 96-well plate assay system to characterize pharmacologically the vanilloid receptor in the dorsal spinal cord of the rat.

Hayes et al. (2000) described the cloning and functional expression of a human orthologue of rat vanilloid receptor-1.

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H.1.1.9.3**Evaluation of Vanilloid Receptor Antagonists****PURPOSE AND RATIONALE**

Several vanilloid receptor antagonists were described, such as capsazepine (Bevan et al. 1992; Walpole et al. 1994) or iodo-resiniferatoxin (Wahl et al. 2001). Kirschstein et al. (1999) described the inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonists.

PROCEDURE

Adult Sprague Dawley rats of both sexes are deeply anesthetized with diethyl ether and rapidly decapitated. The spine is chilled at 4°C in F12 Dulbecco's modified Eagle's medium saturated with carbogen gas and additionally containing 30 mM NaHCO₃, 100,000 units/l penicillin and 100 mg/l streptomycin. Thoracic and lumbar dorsal root ganglions are quickly dissected and freed from connective tissue. Neurons are dissociated in an incubation chamber enriched with carbogen gas at 37°C using collagenase CLS II (5–10 mg/ml, 10–12 min) and trypsin (0.2–1 mg/ml, 10–12 min) dissolved in F12 medium. After trituration (4–6 times with a Pasteur pipette) neurons are plated in 35-mm culture dishes, which also serve as recording chambers, and stored at 37°C in a humidified 5% CO₂ atmosphere before used for electrophysiological recordings.

Only round or oval-shaped neurons without any processes are included in the study. The average of the major and the minor diameter is used to measure the size of oval shaped neurons. Whole cell patch-clamp experiments are performed in carbogen gas saturated F12 medium (pH 7.4) at room temperature using an Axo-patch 200A amplifier (Axon Instruments) in voltage-clamp mode at a holding potential of –80 mV controlled by pCLAMP6 software. Data are also registered on a chart recorder. Patch pipettes are fabricated from borosilicate glass using a horizontal micropipette puller and filled with a solution contain-

ing (in mM) 160 KCl, 8.13 EGTA, 10 HEPES (pH 7.2, $R_{\text{Tip}} = 5.3 \pm 0.2 \text{ M}\Omega$, mean \pm SE). Cell diameter, cross sectional area, and membrane capacitance are measured, and excitability is tested by depolarizing voltage steps for each neuron. Cells lacking a fast inward current with a reversal potential close to the equilibrium potential of sodium followed by a prolonged outward current are excluded from further investigation. Experiments in current-clamp mode are performed to measure the resting membrane potential of each neuron and to investigate single action potentials elicited by short (3 ms) depolarizing current pulses in neurons that are hyperpolarized by constant current injection resulting in membrane potentials between -70 and -80 mV. Inflections in the repolarizing phase are qualitatively detected as second negative peak in the first derivative (dV/dt) of each action potential; the duration of repolarization is quantitatively assessed by the 10–90% decay time.

Applications of $\sim 50 \mu\text{l}$ of heated extracellular solution through a puffing system fixed on a micromanipulator is used to elicit heat-evoked currents. Control measurements with a fast temperature sensor (BAT-12, Physitemp; $\tau = 5$ ms) in place of the neurons are made revealing an effective temperature of $\sim 53^\circ\text{C}$, a rise time of ~ 250 ms, and a decay with a time constant of ~ 20 s. Effects are compared with those of application of the same amount of medium at room temperature. Heat stimuli with or without vanilloid receptor antagonists and control applications at room temperature are repeated 2–10 times, and the elicited currents are averaged. A neuron is considered as heat sensitive when the heat-evoked inward current is significantly greater than any fluctuations caused by superfusion of solution at room temperature. Heating the buffered solution may change its pH, and acid solution of pH 6.2 are known to activate nociceptive dorsal root ganglion neurons (Bevan and Yeats 1991). The pH of a HEPES-buffered solution decreases while heating (e. g., pH 7.1 at 50°C). In contrast, higher temperatures increase the pH of a $\text{NaHCO}_3/\text{CO}_2$ buffer, because the solubility of CO_2 is reduced and thus reverses the HEPES effect. The pH of the F12 medium maximally changes in a range of 7.28–7.52 while heating to 50°C and cooling down to room temperature. The membrane conductance is measured in voltage-clamp mode by hyperpolarizing pulses (5 mV, 10 ms, 50 s^{-1}), and conductance changes are determined at the maximum amplitude of heat evoked currents.

Reversal potentials of heat- and capsaicin-induced currents are measured as described by Liu et al. (1997) using fast depolarizing ramps (-80 to $+30$ mV

in 22 ms every 550 ms). Patch pipettes are filled with a potassium-free solution containing (in mM) 140 CsCl, 10 HEPES, 10 EGTA, and 4 MgCl_2 (adjusted to pH 7.2). Tetrodotoxin ($100 \mu\text{M}$) and nifedipine ($1 \mu\text{M}$) are added to the extracellular solution to block voltage gated Na^+ and Ca^{2+} channels. Capsaicin is dissolved in ethanol, diluted to its final concentration with F12 medium, and applied through the puffing system. Capsazepine (dissolved in DMSO) and ruthenium red are prepared as concentrated stock solutions, diluted to final concentration in F12 medium, and applied either at room temperature or at $\sim 53^\circ\text{C}$. Reversibility of antagonist action is tested by reapplication of heated extracellular solution without any agents.

EVALUATION

Off-line measurements and statistical analysis is done using pCLAMP6 (Axon Instruments) and EXCEL 5.0 (Microsoft). Data are presented as means \pm SE. Treatment effects are statistically analyzed by Student's *t*-test for paired data and χ^2 test for analysis of incidences.

MODIFICATIONS OF THE METHOD

Nagy et al. (1983) described dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat.

Lopshire and Nicol (1998) performed whole-cell and single-channel studies in rat sensory neurons and found a prostaglandin E_2 induced enhancement of the capsaicin elicited current.

Jung et al. (1999) performed patch-clamp experiments in dorsal root ganglion neurons of neonatal rats and concluded that capsaicin binds to the intracellular domain of the capsaicin-activated ion channel.

Nagy and Humphrey (1999) compared the membrane responses of rat sensory neurons to noxious heat and capsaicin, using electrophysiological and ion flux measurements.

Baumann and Martenson (2000) found that extracellular protons both increase the activity and reduce the conductance of capsaicin-gated channels.

Liu et al. (2001) investigated mechanisms underlying capsaicin-mediated inhibition of action potentials and modulation of voltage-gated sodium channels in cultured trigeminal ganglion neurons.

Gunthorpe et al. (2004) identified and characterized a potent and selective vanilloid receptor antagonist isolated via high-throughput screening of a large chemical library in an FLPR-based C^{2+} assay.

For further information on the vanilloid receptor see A.1.1.32.5.

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H.1.2

In Vivo Methods for Testing Central Analgesic Activity

H.1.2.1

General Considerations

Although the *in vivo* methods have been used more extensively in the past, they are still necessary in present research analgesic tests in animals before a compound

can be given to man. Mostly, rodents, such as mice or rats, are used for analgesic tests, but in some instances experiments in higher animals such as monkeys are necessary.

Several methods are available for testing central analgesic activity, such as

- Haffner's tail clip method in mice,
- tail flick or other radiant heat methods,
- tail immersion tests,
- hot plate methods in mice or rats,
- electrical stimulation (grid shock, stimulation of tooth pulp or tail),
- monkey shock titration,
- formalin test in rats.

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H.1.2.2

Haffner's Tail Clip Method

PURPOSE AND RATIONALE

The method was described as early as 1929 by Haffner who observed the raised tail (Straub phenomenon) in mice treated with morphine or similar opioid drugs and found the tail after drug treatment to be less sensitive to noxious stimuli. He already described the high sensitivity of this method to morphine. Since then, the method has been used and modified by many authors.

PROCEDURE

An artery clip is applied to the root of the tail of mice and the reaction time is noted. Male mice (Charles River strain or other strains) with a weight between 18 and 25 g are used. The control group consists of 10 mice. The test compounds are administered subcutaneously to fed mice or orally to fasted animals. The test groups and the control group consist of 7–10 mice. The drug is administered 15, 30 or 60 min prior testing. An artery clip is applied to the root of the tail (approximately 1 cm from the body) to induce pain. The animal quickly responds to this noxious stimuli by biting the clip or the tail near the location of the clip. The time between stimulation onset and response is measured by a stopwatch in 1/10 seconds increments.

EVALUATION

A cut-off time is determined by taking the average reaction time plus 3 times the standard deviation of

the combined latencies of the control mice at all time periods. Any reaction time of the test animals which is greater than the cut-off time is called a positive response indicative of analgesic activity. The length of time until response indicates the period of greatest activity after dosing. An ED_{50} value is calculated at the peak time of drug activity. ED_{50} values found by this method were 1.5 mg/kg s.c. for morphine and 7.5 mg/kg for codeine s.c.

CRITICAL ASSESSMENT OF THE TEST

The test does not need any sophisticated equipment but a skilled, preferably "blind", observer. Peripheral analgesics of the salicylate type are not detected by this test.

MODIFICATIONS OF THE METHOD

Bartoszyk and Wild (1989) described a modification of the original Haffner clip test using pressure on the tail of rats instead of mice. Additionally hyperalgesia was induced by injection of carrageenan suspension into the tail. In this case not only an effect of a nonsteroidal anti-inflammatory agent but also a potentiation by B-vitamins could be shown.

Takagi et al. (1966) published a modification of HAFFNER's method for testing analgesics.

Ossipov et al. (1988) used the Haffner test to compare the antinociceptive effects of intrathecally administered opiates, α_2 -adrenergic agonists, and local anesthetics.

Yanagisawa et al. (1984) described a tail pinch method *in vitro* for testing antinociceptive drugs consisting of an isolated spinal cord, spinal nerve roots and the functionally connected tail of a new-born rat. Changes of electric potential in the ventral root are induced by noxious pressure on the tail. In addition, responses after electric stimulation of the dorsal root were recorded. The authors recommend the method for studying actions of analgesic drugs.

Pinch of the toes of guinea pigs was recommended as a test for opioid analgesics by Collier (1965).

Tail-pinch feeding in rats after intracerebroventricular injection of various opioid antagonists has been used to differentiate opioid receptor subtypes (Koch and Bodnar 1993).

Person et al. (1985) used three different techniques of mechanical tail stimulation (reaction threshold determined with an Analgesymeter at two different cut-off values and HAFFNER's tail clip) to study morphine-caffeine analgesic interaction in rats.

Arndt et al. (1984) studied pain responses (increase of heart rate and arterial pressure, respiratory ef-

fects) to tail clamping in trained unanesthetized spontaneously breathing dogs after administration of fentanyl.

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H.1.2.3

Radiant Heat Method

PURPOSE AND RATIONALE

Originally, the method was developed by Schumacher et al. (1940), Wolff et al. (1940) for quantitative measurements of pain threshold in man against thermal radiation and for evaluation of analgesic activity of opiates. Later on, the procedure has been used by many authors to evaluate analgesic activity in animal experiments by measuring drug-induced changes in the sensitivity of mice or rats to heat stress applied to their tails. The test is very useful for discriminating between centrally acting morphine-like analgesics and non-opiate analgesics.

Mice are placed into cages leaving the tail exposed. A light beam is focused to the proximal third of the tail.

Within a few seconds the animal flicks the tail aside or tries to escape. The time until this reaction occurs is measured.

PROCEDURE

The method was described by Ther, Lindner and Vogel (1963) as a modification of earlier publications (D'Armour and Smith 1941). Groups of 10 mice (NMRI-strain) of both sexes with a weight between 18 and 22 g are used for each dose. Before administration of the test compound or the standard the normal reaction time is determined. The animal is put into a small cage with an opening for the tail at the rear wall. The tail is held gently by the investigator. By opening of a shutter, a light beam exerting radiant heat is directed to the proximal third of the tail. For about 6 s the reaction of the animal is observed by the investigator. The mouse tries to pull the tail away and turns the head. With a switch the shutter is closed as soon as the investigator notices this reaction. Mice with a reaction time of more than 6 s are not used in the test. The escape reaction which is the endpoint of this test can be regarded as a complex phenomenon mediated by the brain. In contrast, the simple tail flick as an endpoint of this test may be mediated as a spinal reflex. Therefore the observation of the escape reaction can be regarded as a true assessment of the influence of the drug on the brain.

The test compounds and the standard are administered either orally or subcutaneously. The animals are submitted to the same testing procedure after 30, 60 and eventually 120 min. For each individual animal the reaction time is noted. Other time intervals can be used according to the question to be investigated.

EVALUATION

There are two possibilities for evaluation:

- The average values of reaction time after each time interval are calculated and compared with the pretest value by analysis of significance.
- At each time interval only those animals which show a reaction time twice as high or higher as the pretest value are regarded as positive. Percentages of positive animals are counted for each time interval and each dose and ED_{50} values are calculated according to LITCHFIELD and WILCOXON.

As standards codeine, pethidine and morphine can be used. The ED_{50} values of these drugs are:

- Codeine 12 mg/kg s.c.
- Pethidine 12 mg/kg s.c.
- Morphine 2 mg/kg s.c.

CRITICAL ASSESSMENT OF THE TEST

The radiant heat test on the tail of mice is very effective to estimate the efficacy and potency of central acting analgesic drugs. With pyrazolones ED_{50} values still can be calculated but these are achieved only with relatively high doses. Compounds like acetylsalicylic acid and phenyl-acetic acids show only slight effects making it impossible to calculate ED_{50} values.

MODIFICATIONS OF THE METHOD

Originally, the method has been described for testing analgesic properties in the rat (D'Armour and Smith 1941, Winter et al. 1954, Harris and Pierson 1964). Goldstein and Malseed (1979) adapted the procedure for utilization in **cats**. The effect of morphine could be antagonized by naloxone in this test. No response to sodium salicylate or pentobarbital was observed. Lutz et al. (1994) used a modification of the rat tail withdrawal test to investigate the structure-activity profile of a series of opioid analgesics. One day before testing, polyethylene tubings were implanted in the femoral vein and externalized behind the neck for intravenous application of test substances.

Various instruments have been described for measuring tail flick latencies by several authors, e.g., Davies et al. 1946; Owen et al. (1981), Isabel et al. (1981), Walker and Dixon (1983), Yoburn et al. (1984), Harris et al. (1988).

Tail flick analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

Green and Young (1951) compared the heat and pressure analgesiometric methods in rats. Mohrland et al. (1983) described an ultrasound-induced tail-flick procedure.

Hargreaves et al. (1988), Costello and Hargreaves (1989), Hylden et al. (1991) exposed the plantar surface of hindpaws of unrestrained rats to a beam of radiant heat applied through the glass floor of a testing chamber. Paw withdrawal latency was automatically recorded by a photocell.

This method was also used by Schuligoi et al. (1994).

Taylor et al. (1997) used this method to investigate the brief (phase 1) and persistent (phase 2) nociceptive responses of rats after injection of dilute formalin into the hindpaw.

Carmon and Frostig (1981) used brief laser induced heat applied to the rat ear for pharmacological testing of analgesics.

Perkins et al. (1993), Perkins and Kelly (1993) used ultra-violet-induced hyperalgesia in rat paw. Female

Sprague-Dawley rats weighing about 100 g were exposed on the plantar surface of one hind paw to UV light (intensity maximum 365 nm, 69 mW/cm²) for 90 s and this was repeated 18 h later. On the following days, each group of rats was placed in a transparent Perspex box and the withdrawal threshold to a focused beam of radiant heat applied to the underside of each hind paw was measured.

McCallister et al. (1986) directed radiant heat to the ears of rabbits and measured ear-withdrawal time.

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H.1.2.4 Hot Plate Method

PURPOSE AND RATIONALE

The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses.

PROCEDURE

The method originally described by Woolfe and MacDonald (1944) has been modified by several investigators. The following modification has been proven to be suitable:

Groups of 10 mice of either sex with an initial weight of 18 to 22 g are used for each dose. The hot plate, which is commercially available, consists of an electrically heated surface. The temperature is controlled for 55° to 56°C. This can be a copper plate or a heated glass surface. The animals are placed on the hot plate and the time until either licking or jumping occurs is recorded by a stop-watch. The latency is recorded before and after 20, 60 and 90 min following oral or subcutaneous administration of the standard or the test compound.

EVALUATION

The prolongation of the latency times comparing the values before and after administration of the test compounds or the values of the control with the experimental groups can be used for statistical comparison using the *t*-test. Alternatively, the values which exceed the value before administration for 50% or 100% can be regarded as positive and *ED*₅₀ values can be calculated.

Doses of 7.5 mg/kg s.c. morphine hydrochloride, 30 mg/kg s.c. codeine hydrochloride, 30 mg/kg s.c. pethidine hydrochloride and 400 mg/kg s.c. phenazone were found to be effective, whereas aspirin showed no effect even at high doses.

CRITICAL ASSESSMENT OF THE TEST

The hot plate test has been used by many investigators and has been found to be suitable for evaluation of centrally but not of peripherally acting analgesics. Mice as well as rats have been used. The method has the drawback that sedatives and muscle relaxants (Woolfe and MacDonald 1944) or psychotomimetics (Knoll 1967) cause false positives, while mixed opiate agonists-antagonists provide unreliable results. The validity of the test has been shown even in the presence of substantial impairment of motor performance (Plummer et al. 1991). Mixed opiate agonists-antagonists can be evaluated if the temperature of the hot plate is lowered to 49.5°C (O'Callaghan and Holtzman 1975; Zimer et al. 1986).

MODIFICATIONS OF THE METHOD

O'Neill et al. (1983) described an automated, high-capacity method for measuring jump latencies on a hot plate. A hot-plate test with increasing temperature was recommended by Tjølsen et al. (1991).

Hot plate analgesy meters are commercially available (e. g., IITC Life Science, Woodland Hills, CA, USA).

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H.1.2.5**Tail Immersion Test****PURPOSE AND RATIONALE**

The method has been developed to be selective for morphine-like compounds. The procedure is based on the observation that morphine-like drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 55°C.

PROCEDURE

Young female Wistar rats (170–210 g body weight) are used. They are placed into individual restraining cages

leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55°C. Within a few seconds the rat reacts by withdrawing the tail. The reaction time is recorded in 0.5 s units by a stopwatch. After each determination the tail is carefully dried. The reaction time is determined before and periodically after either oral or subcutaneous administration of the test substance, e. g., after 0.5, 1, 2, 3, 4 and 6 h. The cut off time of the immersion is 15 s. The withdrawal time of untreated animals is between 1 and 5.5 s. A withdrawal time of more than 6 s therefore is regarded as a positive response.

EVALUATION

ED_{50} values can be calculated for each compound and time response curves (onset, peak and duration of the effect) be measured. All the morphine-like analgesics have been shown to be active at doses which do not produce gross behavioral changes. For example, an ED_{50} of 3.5 mg/kg s.c. for morphine and an ED_{50} of 1.7 mg/kg s.c. methadone was found. Acetylsalicylic acid at a dose of 640 mg/kg p.o., phenylbutazone at a dose of 160 mg/kg s.c. as well as nalorphine at a dose of 40 mg/kg s.c. were inactive.

CRITICAL ASSESSMENT OF THE TEST

The test is useful to differentiate central opioid like analgesics from peripheral analgesics.

MODIFICATIONS OF THE METHOD

Ben-Bassat et al. (1959) described the receptacle method in mice. Each mouse was inserted in a conoid paper receptacle with its tail protruding, the cone being closed by a stapler. The protruding tail was entirely immersed in a water bath (58°C) and the time until withdrawal of the tail was measured by a stop watch.

Pizziketti et al. (1985) modified the tail immersion test in rats in this way that they used a 1:1 mixture of ethylene-glycol and water **cooled** to a temperature of minus 10°C as noxious stimulus. Linear dose-response curves were found with levo-methadone and morphine. Low ceiling effects or curvilinear dose-response curves were obtained with narcotic agonist-antagonist analgesics such as pentazocine. Diazepam and aspirin were inactive.

Tiseo et al. (1988) could show that the endogenous kappa agonist dynorphin A was inactive in the rat tail immersion test at 55°C, but gave dose-response curves in the cold water version of the test.

Abbott and Melzack (1982) examined the effects of brainstem lesions on morphine analgesia using the formalin test which produced moderate pain that lasted about 2 h, and the tail-flick hot water-immersion test which measured brief threshold-level pain.

Abbott and Franklin (1986) used two forms of the rat tail flick test: In the restrained form of the test rats were placed in wire restraining tubes from 10 min before drug injection till the end of the test. In the unrestrained form of the test rats were left free in their home cages and handheld during each test for approximately 30 s. Responses to the thermal pain stimulus were assessed by the latency with which the rat removed its tail from 55°C water. Two types of morphine analgesia have been postulated in animals: One type, exemplified in rats that are restrained during tail flick testing, is sensitive to an interaction between morphine and brain 5-HT, the level of which is elevated by restrained stress (Kelly and Franklin 1984).

Luttinger (1985) determined the antinociceptive activity of drugs using different water temperatures in a tail-immersion test in mice. The results roughly paralleled the differences in the severity of pain for which various analgesics are effective.

Dykstra et al. (1986, 1987) described a tail withdrawal procedure for assessing analgesic activity in **Rhesus monkeys** by immersion of the tail into water of 55°C. This procedure was used by Rothman et al. (1989) to determine the pharmacological activities of optically pure enantiomers of the κ opioid agonist, U50,488, and its cis diastereomer.

Using this method, Ko et al. (1999) found that activation of peripheral κ opioid receptors inhibits capsaicin-induced nociception in Rhesus monkeys.

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H.1.2.6

Electrical Stimulation of the Tail

PURPOSE AND RATIONALE

Since the tail of mice is known to be sensitive to any stimulus, a method of electrical stimulation has been described as early as 1950 by Burn et al. The stimulus can be varied either by the duration of the electric shock or by an increase in the electric current.

PROCEDURE

As described by Kakunaga et al. (1966), male mice with a weight of 20 g are placed into special cages. A pair of alligator clips is attached to the tail whereby the positive electrode is placed at the proximal end of the tail. Rectangular wave pulses from a constant voltage stimulator at an intensity of 40–50 V are applied. The frequency of the stimulation is 1 shock/s, and the

pulse duration 2.5 ms. The normal response time range of the stimuli is 3–4 s. Following administration of the drug, the response time is registered at 15 min intervals until the reaction time returns to control levels.

EVALUATION

The data for each animal are plotted with reaction times on the ordinate and time intervals following administration on the abscissa. The area under the time response curve is calculated. In control animals the reaction time remains fairly constant and the area under the curve is approximately zero. Effects of morphine at 5 mg/kg s.c. and meperidine 30 mg/kg s.c. could easily be demonstrated.

CRITICAL ASSESSMENT OF THE TEST

The effect of central analgesics can be clearly demonstrated, however also the activity of peripheral analgesics given at higher doses can be detected.

MODIFICATIONS OF THE METHOD

Vidal et al. (1982) measured the thresholds of 3 nociceptive reactions (tail withdrawal, vocalization, vocalization afterdischarge) following electrical stimulation of the tail.

A variation of the test has been introduced by Yanaura et al. (1976), using ultrasonic stimulation instead of electric stimulation. The method is considered to be fast, simple, and precise. The stimulus can be applied repeatedly without causing injury to the tissue. A vocalization test in rats with electrical stimulation of the tail has been described by Hoffmeister (1968).

Ludbrook et al. (1995) described a method for frequent measurement of sedation and analgesia in sheep using the response to a ramped electrical stimulus. Sheep were placed in a canvas sling in their metabolic crates to allow their limbs to partially bear weight in order to minimize spontaneous limb movements. Two needles were placed subcutaneously 0.5 cm apart in the anterior aspect of the lower third of the sheep's hind and connected to the nerve stimulator. The current ramp rate was set at one mA per sec. As soon as limb withdrawal was observed, the stimulus was switched off and the highest current and ramp duration were recorded.

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H.1.2.7

Grid Shock Test

PURPOSE AND RATIONALE

The electric grid shock test in mice has been described by Blake et al. (1963) as a modification of an earlier approach (Evans 1962) to measure the analgesic properties by the “Flinch-jump” procedure in rats.

PROCEDURE

Male mice with a weight between 18 and 20 g are individually placed into clear plastic chambers. The floor of the box is wired with tightly strung stainless steel wire, spaced about 1 mm apart. The stimulus is given in the form of square wave pulses, 30 cycles per second with a duration of 2 ms per pulse. The output of the stimulator has to be connected to alternate wires of the grid. A fixed resistance is placed in series with the grid and in parallel to an oscilloscope to allow calibration in milliamperes. With increasing shock intensities the mice flinch, exhibit a startling reaction, increase locomotion or attempt to jump. The behavior is accurately reflected on the oscilloscope by marked fluctuations of the displayed pulse and defined as pain threshold response. Pain thresholds are determined in each individual mouse twice before administration of the test drug and 15, 30, 60, 90 and 120 min after dosing. Groups of 10 animals are used for control and for the test drugs.

EVALUATION

The current as measured in milliamperes is recorded for each animal before and after administration of

the drug. The average values for each group at each time interval are calculated and statistically compared with the control values. Placebo treated controls show a slight increase of threshold over time. Morphine sulfate in a dose of 10 mg/kg p.o. but also acetylsalicylic acid in a dose of 200 mg/kg p.o. definitely increase the threshold.

CRITICAL EVALUATION OF THE METHOD

The modification of the method as described by Blake et al. (1963) showed an effect not only of morphine but also of acetylsalicylic acid which is not easily picked up by other tests based on stimulation by physical means.

MODIFICATIONS OF THE TEST

Weiss and Laties (1961) in a “fractional escape” procedure trained animals to press a lever to reduce the intensity of shock delivered continuously through the floor grids of the experimental chamber. Each time, the rat depresses the lever, it reduces the intensity of the shock. An external timer is programmed to increase the intensity of the shock every few seconds. If the animal fails to press the lever, the shock continues to increase in intensity until lever-pressing behavior drives it down. Thus, the level of shock fluctuates depending on the rat’s lever pressing. The action of an analgesic in altering the level of shock which the rat will “tolerate” can then be measured by comparing the average level at which the rat maintains the shock under control conditions with the average level at which the rat maintains the shock during treatment.

Painful stimulation of the paws of mice placed into cages equipped with metal bands for electrical stimulation was described by Charlier et al. (1961) as “pododolorimetry”.

A modification of the jump-flinch technique for measuring pain sensitivity in rats based on four categories of responses was described by Bonnet and Peterson (1975).

Eschaliere et al. (1988) described an automated method to analyze vocalization of unrestrained rats submitted to noxious stimuli.

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the phenomenon of licking occurs. In some cases, the current has to be increased and then to be decreased again in order to find the appropriate threshold. For assessing the basic value, the threshold is determined 3 times in each animal. Each animal serves as its own control. For testing analgesic activity of a new drug and determination of an ED_{50} 8 to 10 animals are used for each dose of the analgesic. The test substance is either injected intravenously or given orally by gavage. The threshold as the indicator of the antinociceptive effect is determined again after 15, 30, 60 and 120 min. The animals serve as their own controls. Threshold current is determined again 5, 15, 30, 45 and 60 min after intravenous application and 15, 30, 60 and 120 min after oral application.

EVALUATION

For screening procedures the increase of threshold, expressed in mV, is the indicator of intensity and duration of the analgesic effect. For determination of the ED_{50} , 8 to 10 rabbits are used for each dose, using 3 doses, which provided effects between 10 and 90%. An antinociceptive effect is defined as an increase of the threshold versus the initial control by a factor of 2 or more.

CRITICAL ASSESSMENT OF THE METHOD

Central analgesics, especially opioid agonists, have been found to be very active in this test. Compared with other tests for central analgesic activity, like the hot plate test in mice, the tests result in lower ED_{50} -values indicating a high sensitivity of the method. In addition, non-opiate analgesics like ketamine and peripheral analgesics like pyrazolone derivatives gave a positive response.

MODIFICATIONS OF THE METHOD

The method has been performed primarily in rabbits (Hertle et al. 1957; Hoffmeister 1962, 1968; Piercey and Schröder 1980), but also **dogs** (Koll and Fleischmann 1941; Skinkle and Tyers 1979) and **cats** (Mitchell 1964) have been used.

Among several methods in different species, Fleisch and Dolivo (1953) found the electrical stimulation of the tooth pulp in the rabbit as the only satisfactory method to test the efficacy of different analgesic drugs.

The effects of tooth pulp stimulation in the thalamus and hypothalamus of the **rat** have been investigated by Shigena et al. (1973).

The method has been adapted for freely moving rats (Steinfels and Cook 1986). Medium effective doses could be determined for $\bar{\iota}$ and $\hat{\epsilon}$ agonists. Non-steroidal

H.1.2.8

Tooth Pulp Stimulation

PURPOSE AND RATIONALE

The method has been first described by Kohl and Refert (1938) and by Ruckstuhl and Gordanoff (1939) for testing central analgesic activity in rabbits and has since applied by several authors to various animal species. Stimulation of the tooth pulp induces characteristic reactions, such as licking, biting, chewing and head flick which can be observed easily.

PROCEDURE

Rabbits of either sex with an weight between 2 and 3 kg are anaesthetized with 15 mg/kg thiopental or 0.2 mg/kg fentanyl-citrate intravenously. Pulp chambers are exposed close to the gingival line in the lateral margins of the two front upper incisors with a high-speed dental drill. On the day of the experiment, clamping electrodes are placed into the drilled holes. After an accommodation period of 30 min stimulation is started to determine the threshold value. The stimulus is applied by rectangular current with a frequency of 50 Hz and a duration of the stimulus of 1 s. The electrical current is started with 0.2 mA and increased until

anti-inflammatory drugs were also effective in this test procedure, but the slopes of the dose-response curves for these compounds were lower than for the opioid analgesics. Microinfusion of bradykinin solution onto the tooth pulp of unrestrained rats was described by Foong et al. (1982) as a reliable method for evaluating analgesic potencies of drugs on trigeminal pain.

Kidder and Wynn (1983) described an automatic electronic apparatus for generating and recording a ramp stimulus for analgesia testing.

Thut et al. (1995) used the rabbit tooth-pulp assay to quantify efficacy and duration of antinociception by local anesthetics infiltrated into maxillary tissues.

Shyu et al. (1984) studied the role of central serotonergic neurons in the development of dental pain in the **monkey**.

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H.1.2.9

Monkey Shock Titration Test

PURPOSE AND RATIONALE

Generally, analgesic tests in rats and mice result in correlation with the analgesic activity of a drug in man. To clarify the mode of action in more detail and to find a suitable dose for therapy in man, experiments in monkeys may be necessary.

PROCEDURE

This test has been recommended by Weiss and Laties (1958) and later developed further by several authors. The monkeys are seated in restraining chairs. Electrical current is delivered by a Coulbourn Instrument Programmable Shocker through electrodes coupled to two test tube clamps which are attached to a shaved portion of the tail. The current ranges from 0 to 4 mA through 29 progressive steps. The monkey presses a bar to interrupt the shock. A stable baseline shock level is established for each monkey on the day prior to drug administration. After drug administration shock titration activity is rated according to the change in maximum level of median shock intensity attained for drug as compared to control levels. Doses of 3.0 mg/kg i.m.

morphine, 1.7 mg/kg i.m. methadone and 10 mg/kg i.m. pentazocine were found to be effective.

CRITICAL ASSESSMENT

The monkey shock titration test may be used for final evaluation of a new compound before administration to man. For screening activities the procedure can not be recommended since the test is too time consuming and the apparatus too complicated. Furthermore, higher animals such as monkeys should only be used if absolutely necessary.

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H.1.2.10

Formalin Test in Rats

PURPOSE AND RATIONALE

The formalin test in rats has been proposed as a chronic pain model which is sensitive to centrally active analgesic agents by Dubuisson and Dennis (1977).

PROCEDURE

Male Wistar rats weighing 180–300 g are administered 0.05 ml of 10% formalin into the dorsal portion of the front paw. The test drug is administered simultaneously either sc. or orally. Each individual rat is placed into a clear plastic cage for observation. Readings are taken at 30 and 60 min and scored according to a pain scale. Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw. Analgesic response or protection is indicated

if both paws are resting on the floor with no obvious favoring of the injected paw.

EVALUATION

Using various doses, ED_{50} values for protection can be calculated. Doses of 1.7 mg/kg morphine s.c. and 15 mg/kg s.c. pethidine were found to be effective.

CRITICAL ASSESSMENT

The formalin test identifies mainly centrally active drugs, whereas peripherally acting analgesics are almost ineffective. Therefore, the formalin test may allow a dissociation between inflammatory and non-inflammatory pain, a rough classification of analgesics according to their site and their mechanism of action (Chau 1989). Cowan (1990) underlined the aspect that the formalin-test is a model of chronic pain whereas most other methods measure only the effect on acute pain.

MODIFICATIONS OF THE METHOD

Murray et al. (1988) used mice instead of rats. They injected 0.020 ml of 5% formalin solution into the subplantar region of the hind paw. Morphine at a dose of 2.1 mg/kg s.c. and pentazocine at a dose of 23.8 mg/kg s.c. were active whereas the cyclooxygenase inhibitor zomepirac was inactive even at a dose of 100 mg/kg s.c.

Hunskar et al. (1986), Hunskar and Hole (1987) injected a small amount of formalin (20 μ l of 1% solution) under the skin of the dorsal surface of the right hind paw of mice. A biphasic response with an early (0–5 min) and a late (20–30 min) phase with high licking activity was observed. Centrally acting analgesics were active in both phases, whereas non-steroidal anti-inflammatory drugs and corticosteroids inhibited only the late phase. Acetylsalicylic acid and paracetamol were antinociceptive in both phases.

Shibata et al. (1989) again used lower concentrations of formalin (0.025 ml of 0.5% formalin solution) and also mice instead of rats. They found a characteristic biphasic pain response. Centrally acting drugs such as morphine inhibited both phases, whereas according to their data peripherally acting drugs such as acetylsalicylic acid, oxyphenylbutazone and corticosteroids inhibited only the second phase.

Abbott et al. (1995) used the formalin test for scoring properties of the first and second phases of the pain response in rats.

Abbadie et al. (1997) determined the pattern of c-fos expression in the rat spinal cord to study the two phases of the formalin test.

Clavelou et al. (1989), Dallel et al. (1995). Gilbert et al. (2001) used a modification of the formalin test for assessing pain and analgesia in the **orofacial region of the rat**. After injection into the upper lip, pain intensity was evaluated by the animal's behavior of rubbing of the injected area. A subcutaneous injection of 0.05 ml of 0.92% formaldehyde solution was made into the upper lip, just lateral to the nose. Following injection, the rat was immediately brought back in a test box equipped with a videocamera for a 45 min observation period. The recording time was divided into 15 blocks of 5 min and a pain score was determined for each block, by measuring the number of seconds that the animals spent rubbing the injected area with the ipsilateral fore- or hindpaw. The animals were sacrificed after the end of the experiment to avoid unnecessary suffering.

Tjølsen et al. (1992) attributed the early phase to C-fibre activation, whereas the late phase appeared to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord.

Alreja et al. (1984) used the formalin test for assessing pain in **monkeys** after volunteering of one of the authors to carry out the same procedure on himself.

Corrêa and Calixto (1993) studied the participation of B₁ and B₂ kinin receptors in the formalin-induced nociceptive response in the **mouse**. Pain response was increased after ACE-inhibition and decreased by bradykinin receptor antagonists.

Herman and Felinska (1979) proposed a rapid test for screening of narcotic analgesics in mice by evaluation of behavioral symptoms after subcutaneous injection of EDTA.

Legat et al. (1994), Dumas et al. (1997) induced hyperalgesia in rats by subplantar injection of collagenase (100 µg in 100 µl saline) and rated the behavioral reactions after treatment with analgesics according to a modified formalin-test.

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H.1.2.11

Neuropathic Pain

H.1.2.11.1

General Considerations

Partial injury to somatosensory nerves sometimes causes causalgia in humans. Causalgia is characterized by spontaneous burning pain combined with hyperalgesia and allodynia and usually follows an incomplete peripheral nerve injury. Allodynia, a pain sensation due to normally innocuous stimulation, is a particularly troublesome symptom in patients. Neuropathic pains are classified according to either the etiological diagnosis of the neuropathy (e. g., painful diabetic neuropathy, post-herpetic neuralgia, post-traumatic neuralgia, etc.), or the anatomical lesion (e. g., central pain, peripheral neuralgia). See Hansson and Dickenson (2005). Various animal models are described to study neuropathic pain. See below.

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H.1.2.11.2

Chronic Nerve Constriction Injury

PURPOSE AND RATIONALE

Bennet and Xie (1988) described a peripheral neuropathy due to nerve constriction in the rat that produces disorders of pain sensation like those seen in man. This method with slight modifications was used by Davar et al. (1991), Mao et al. (1992), Munger et al. (1992), Yamamoto and Yaksh (1992), Tal and Bennet (1993) and reviewed by Bennett (1993).

PROCEDURE

Anesthesia is induced in male Sprague-Dawley rats by inhalation with halothane 4% and maintained at a concentration of 2–3% as needed. After a local incision, the biceps femoralis of each leg is bluntly dissected at mid thigh to expose the sciatic nerve. Each nerve is then mobilized with care taken to avoid undue stretching. Four 4–0 chromic gut sutures are each tied loosely with a square knot around the right sciatic nerve. The left sciatic nerve is only mobilized. Both incisions are

closed layer to layer with silk sutures and the rats allowed to recover. During the next days, the animals show a mild eversion of the affected paw and a mild-to-moderate degree of foot drop.

The thermal nociceptive threshold is measured according to the method of Hargreaves et al. (1988), (see H.1.2.3). The rats are placed beneath a clear plastic cage (10 × 20 × 24 cm) upon an elevated floor of clear glass. A radiant heat source (halogen projector lamp) is placed beneath the glass floor on a movable holder and positioned such that it focuses at the plantar area of one hind paw. The time interval between the application of the light beam and the brisk hind paw withdrawal response is measured to the nearest 0.1 sec.

The maximum hyperesthesia occurs between 7 and 14 days after nerve ligation. Before intrathecal injection of the drug or vehicle, the hind paws are tested 3 times alternatively with 5-min intervals as the baseline data. The left and right test sequence is carried out at 5, 15, 30, 60 and 90 min after injection.

EVALUATION

The mean \pm SEM of the paw withdrawal latency (PWL) is plotted. To analyze the magnitude of hyperesthesia, the difference score (DS) is calculated by subtraction the maximum PWL of the control side (left side) from the maximum PWL of the affected side (right side). Maximum PWL is defined as the PWL that was the maximum during the first 30 min after injection. To analyze the drug effects in hyperesthetic rats, the dose is plotted against the change in DS (post-drug difference score minus pre-drug difference score).

MODIFICATIONS OF THE METHOD

The chronic constriction injury (CCI) model according to Bennett and Xie (1988) has been used by several authors: Sotgiu and Biella (1998), Toda et al. (1998), Blackburn-Munro and Jensen (2003), Keay et al. (2004), Bingham et al. (2005), Bomholt et al. (2005), Costa et al. (2005), and Howard et al. (2005).

Sotgiu et al. (1996) performed laminectomy from L1 to S2 in anesthetized rats with sciatic chronic constriction injury. For extracellular recording, two tungsten microelectrodes were positioned under a dissecting microscope on the surface of the spinal cord at L2 and L5–L6 level ipsilaterally to the injured nerve, and were advanced at steps of 2 μ m. Neuronal activity was conventionally recorded and then digitized; frequency histograms were constructed by computer programs. The search stimulus for dorsal horn neurons at L2 and L5–L6 segments was the electrical stimulation of saphenous and sciatic nerve peripheral territories. Nat-

ural stimuli (brushing of the skin) and noxious stimuli (calibrated pinching) were defined. After the responses to saphenous stimuli in the neurons were recorded, a small pad of gel-foam soaked with 0.5 ml lidocaine was placed around the intact epineurium proximally to the ligatures on the sciatic nerve. The saphenous stimulation was repeated during the block and after complete recovery of the preblock baseline activity. In this way, the effect of the local anesthetic on the spontaneous activity and on the response to a noxious stimulus could be evaluated.

The first animal model of painful neuropathy was reported by Wall et al. (1979a, b). The sciatic nerve of rats or mice was sectioned and either tied or implanted in a polyethylene tube sealed at its far end. Moreover, in one modification also the saphenous nerve was cut, such that the hind paw was completely denervated. This procedure, which is known as the neuroma model, is believed to replicate the human syndromes seen after amputation (phantom pain) or after nerve transection in an intact limb (anesthesia dolorosa). Within several days, the animals begin to self-mutilate the hindpaw on the side of the nerve transection: a behavior named 'autotomy'.

Seltzer et al. (1990) ligated only one-half of the sciatic nerve in rats unilaterally. The withdrawal thresholds to repetitive von Frey hair stimulation at the plantar side were decreased bilaterally as were the withdrawal thresholds to CO₂ laser heat pulses. The contralateral phenomena resemble the "mirror image" pains in humans with causalgia.

This "partial sciatic nerve injury model" has been used by several authors (Malmberg and Basbaum 1998; Lindenlaub and Sommer 2000; Bingham et al. 2005). Patel et al. (2001) studied the effects of GABA_B agonists and gabapentin on mechanical hyperalgesia in models of neuropathic (partial sciatic ligation) and inflammatory (Freund's complete adjuvant) pain in the rat and the inhibitory action on spinal transmission *in vitro*.

Hofmann et al. (2003) described the tibial nerve injury model in rats as a surgically uncomplicated model of neuropathic pain based on unilateral transection (neurotomy) of the tibial branch of the sciatic nerve.

Walczak et al. (2005) characterized the saphenous nerve partial ligation in rats as a model of neuropathic pain.

Kim and Chung (1992) described an experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Either both the L₅ and L₆ spinal nerves or the L₅ spinal nerve alone on one side of the rat were tightly ligated. A modified ver-

sion of this technique was used by LaBuda and Little (2005) and by Bertorelli et al. (2005).

DeLeo et al. (1994) performed cryoneurolysis of the sciatic nerve in the rat using a cryoprobe cooled to -60°C in a 30/5/30 s freeze-thaw-freeze sequence. Autotomy was observed after 4–14 days.

Coderre et al. (2004) produced a neuropathic-like pain syndrome in rats following prolonged hind paw ischemia and reperfusion, creating an animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy), called **chronic post-ischemia pain**. A tourniquet ring was placed on one hindlimb of an anesthetized rat just proximal to the ankle joint for 3 h, which was removed prior to termination of anesthesia to allow reperfusion. Rats exhibited hyperemia and edema/plasma extravasation of the ischemic hind paw for a period of 2–4 h after reperfusion. Hyperalgesia to noxious mechanical stimulation (pin prick) and cold (acetone exposure), as well as mechanical allodynia to innocuous mechanical stimulation (von Frey hairs) are evident in the affected hindpaw as early as 8 h after reperfusion, and extend for at least 4 weeks.

Mice that lack protein kinase C gamma (PKC γ) displayed normal response to acute pain stimuli, but they almost completely failed to develop a neuropathic pain syndrome after partial sciatic nerve section, and the neurochemical changes that occurred in the spinal cord after nerve injury were blunted (Malmberg et al. 1997).

Shimoyama et al. (2002) developed a **mouse model of neuropathic cancer pain** by inoculating Meth A sarcoma cells in the immediate proximity of the sciatic nerve in BALB/c mice. The tumor grows predictably with time and gradually compresses the nerve, thereby causing nerve injury. Time courses of thermal hypersensitivity and mechanical sensitivity to von Frey hairs were determined and signs of spontaneous pain were evaluated. The authors compared this model with the chronic constriction model.

Panesar et al. (1997) and Campbell et al. (1998) studied mechanical hyperalgesia associated with partial peripheral ligation in the **guinea pig**.

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H.1.2.11.3

Peripheral Nerve Injury Model

PURPOSE AND RATIONALE

In addition to chronic ligation techniques, nerve dissection and nerve crush models have been used to study neuropathic pain. Nerve crush injury was described by Decosterd et al. (2002).

PROCEDURE

Male Sprague Dawley rats weighing 200–250 g were anesthetized with halothane (1.5%–3%). The left sciatic nerve was exposed at the mid-thigh level and crushed by a pair of hemostat forceps with smooth protective pads that were placed perpendicularly to the

sciatic trunk for 30 s (Bester et al. 2000). Muscle and skin were closed in two layers.

Animals were habituated to the tester, the environment and the handling procedures prior to commencement of the testing. A calibrated von Frey monofilament was applied five times until it bent on the lateral dorsal side of the hindpaw that is in crushed nerve territory or the intact sural nerve, ipsilateral to the nerve lesion. A series of monofilaments (1.0–200.0 g) were applied and the test started with the lowest force filament. The mechanical withdrawal threshold corresponds to the minimum force required to elicit a reproducible flexor withdrawal movement after application of the von Frey hairs. Once baseline threshold was determined, eight light strokes were applied manually at 1 Hz to the dorsum of the paw at 5-min intervals, for 2 h. The mechanical withdrawal threshold was recorded immediately after the application of the light touch, at the same interval of 5 min during the whole test period.

EVALUATION

Data are expressed as mean \pm SEM of the recorded mechanical withdrawal threshold in grams, or as a percentage of the baseline value. The differences between groups were analyzed with analysis of variance (ANOVA) two-way repeated measures.

MODIFICATIONS OF THE METHOD

Vogelaar et al. (2004) described sciatic nerve regeneration in mice and rats. Under anesthesia, the sciatic nerve was carefully exposed. At a point immediately distal from the gluteus maximus muscle, the nerve was crushed for 30 s using a hemostatic forceps. The animals were followed for 70 (rats) and 32 (mice) days and functional recovery of sciatic nerve function was monitored by the foot reflex withdrawal test, locomotor pattern, and mechanical withdrawal thresholds.

Rodrigues-Filho et al. (2003, 2004) published the technique of avulsion injury of the rat brachial plexus that triggers hyperalgesia and allodynia in the hindpaws. Male Wistar rats weighing 250–300 g were anesthetized by chloralose i.p. The brachial plexus was approached through a horizontal incision parallel to the clavicle, running from the sternum to the axillary region. The pectoralis major muscle was displaced, leaving the cephalic vein intact. The subclavian vessels were located and the lower trunk dissected and crushed three times for 5 s using microsurgical forceps. At the end of this procedure, the nerve was completely flattened and transparent. The tissue layers were then brought together and the skin closed with silk sutures.

For assessment of mechanical hyperalgesia, mechanical thresholds were measured in the hindpaws with an analgesymeter (Ugo Basile, Italy) according to the method of Randall and Sellitto (1957). Furthermore, thermal hyperalgesia, mechanical allodynia, and cold allodynia were measured in the hindpaws, as well as grasping force in the forepaws.

Sweitzer et al. (2001) studied prevention of allodynia induced by transection of the L5 spinal nerve in rats.

Devor et al. (2005) studied heritability of symptoms in the neuroma model of neuropathic pain.

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H.1.2.11.4

Spared Nerve Injury Model

PURPOSE AND RATIONALE

In addition to the above-described models of peripheral neuropathic pain [chronic constriction injury model according to Bennett and Xie (1988), partial ligation according to Seltzer et al. (1990), segmental spinal nerve ligation according to Kim and Chung (1992)], Decosterd and Woolf (2000) described **spared nerve injury** as an animal model of persistent peripheral neuropathic pain.

PROCEDURE

Adult male Sprague Dawley rats were used. Under halothane (2%) anesthesia the skin on the lateral surface of the thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. The procedure comprises an axotomy and ligation of the tibial and common peroneal nerves leaving the sural nerve intact. The common peroneal and the tibial nerves were tightly ligated with 5.0 silk and sectioned distal to the ligation, removing 2–4 mm of the distal nerve stump. Care was taken to avoid any contact with or stretching of the intact sural nerve. Muscle and skin were closed in two layers. Behavior testing was performed only after a period of at least 1 week.

For testing mechanical allodynia, animals were placed on an elevated wire grid and the plantar surface of the paw stimulated with a series of ascending force von Frey filaments. The threshold was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive stimuli. The lateral and medial plantar surface of the paw as well as its dorsal surface were tested.

For testing mechanical hyperalgesia, a pin prick test was performed using a safety pin. The lateral part of the plantar surface of the paw was briefly stimulated at an intensity to indent but not penetrate the skin. The duration of paw withdrawal was recorded.

For testing cold allodynia, a drop of acetone solution was delicately dropped onto the lateral plantar surface of the paw, using a blunt needle connected to a syringe without touching the skin. The duration of the withdrawal response was recorded.

The lateral plantar surface was exposed to a beam of radiant heat through a transparent Perspex surface (Hargreaves et al. 1988). The withdrawal latency and duration were recorded. The heat stimulation was repeated 3 times at an interval of 5–10 min for each paw and the mean calculated.

EVALUATION

Results were presented as mean \pm SEM. The data were analyzed by one-way ANOVA and the non-parametric Wilcoxon matched-pairs signed-rank tests.

MODIFICATIONS OF THE METHOD

The rat spared nerve injury model has been used by several authors: Blackburn-Munro and Jensen (2003), Rode et al. (2005), and Howard et al. (2005).

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H.1.2.11.5**Spinal Cord Injury****PURPOSE AND RATIONALE**

Spinal cord injuries result in a devastating loss of function. Chronic central pain syndromes frequently develop in the majority of affected patients. Several attempts have been made to find animal models of this situation. Xu et al. (1992), Hao and Xu (1996), Hao et al. (1998a, 1998b, 2000), Wu et al. (2003), and Colpaert et al. (2004) performed studies in rats after ischemic spinal cord injury photochemically induced by laser irradiation.

PROCEDURE

Female Sprague Dawley rats were anesthetized with 300 mg/kg chloral hydrate i.p. and one jugular vein was cannulated. Vertebrae T11–L2 were exposed after a midline incision of the skin on the back. The animals were positioned beneath a tuneable argon ion laser (Innova, Model 70, Coherent Laser Production Division) and irradiated with a knife edge beam, which was used to cover the single T13 vertebra with an average power of 0.16 W for 10 min. No laminectomy was performed. Immediately before the irradiation, erythrosine B (Red No. 3, Aldrich-Chemie) was injected intravenously in 0.9% saline at a dose of 32.5 mg/kg. Since erythrosine B is rapidly metabolized, the injection at this dose was repeated at 5-min intervals during the irradiation in order to maintain an adequate blood concentration.

Erythrosine B has an optimal absorption wavelength similar to that of the laser light. When it receives this light, a photochemical reaction occurs inside the spinal cord blood vessel where the laser is aimed. One of the reaction products accumulates in the vessel, injuring the endothelial layer and causing platelet release and coagulation and, thus, ischemia. After irradiation the incision was closed and the animals were kept warm for 2 h. The bladders were emptied manually 2–3 times a day until normal function was regained.

A set of calibrated von Frey hairs was used to test the vocalization threshold in response to graded mechanical pressure ranging from 0.021 to 410.0 g. During the test the rats were gently restrained in a standing position by the experimenter and the von Frey hair was pushed onto the skin until the filament became bent. The frequency of the stimulation was about every 3–4 s per stimulus and at each intensity. The stimuli were applied 5–10 times. The pressure which induced consistent vocalization (>75% response rate) over a relatively large skin area was considered to be the pain threshold.

EVALUATION

Rats were randomized for different treatment groups. The data are expressed as medians and variability as mean \pm SEM. Data were analyzed with Kruskal-Wallis one-way ANOVA followed by Wilcoxon signed ranks test or by Mann-Whitney *U*-test.

MODIFICATIONS OF THE METHOD

Christensen et al. (1996), Christensen and Hulsebosch (1997a, 1007b), and Bennett et al. (2000a, 2000b) used a rodent spinal hemisection model of spinal cord injury in which mechanical and thermal allodynia develop by 24 days after injury.

Male Sprague Dawley rats (175–200 g) were deeply anesthetized by i.p. injection of 75 mg/kg ketamine and 15 mg/kg xylazine. The spinal cord was hemidisectioned at T13 on the left side by the following procedure: after palpation of the dorsal surface to locate the cranial borders of the sacrum and the dorsal spinous processes of the lower thoracic and lumbar vertebrae, the T11, T12, and T13 laminae were determined by locating the last rib, which attaches the cranial end of the T13 vertebrae. The surgical field was shaved and prepared with povidone-iodine, and a longitudinal incision was made exposing several segments. A laminectomy was performed at vertebral level T11, the lumbar spinal cord was identified with the accompanying dorsal vessel, and the spinal cord was hemisectioned at T13, cranial to the L1 dorsal root entry zone, with a scalpel blade without damage to the major dorsal

vessel or vascular branches. The musculature and the fascia were then sutured and the skin was apposed by autoclips.

Siddall et al. (1995) and Drew et al. (2004) described mechanical allodynia following contusion injury of the rat spinal cord. Contusive spinal cord injury was produced in anesthetized Wistar rats weighing 200–300 g. Laminectomy was performed at the vertebral thoracic T10 level to expose a 3-mm window over the dorsal spinal cord and adjacent rostral and caudal spinous processes were clamped to stabilize the spine. A brass guide tube 15 cm in length was positioned perpendicularly above the exposed cord and a cylindrical 10 g steel weight (2 mm in diameter) with a rounded tip was suspended within the tube 2 cm above the cord surface. The weight was held within the tube by a metal pin. Removing the pin and allowing the weight to drop onto the exposed cord produced spinal cord injury at the T12–T13 segmental level. The wound was closed in layers and antibiotics were administered.

Abraham et al. (2001) and Caudle et al. (2003) described excitotoxic spinal cord injury induced by intraspinal injection of quisqualic acid.

Zochodne et al. (1994) induced a segmental chronic pain syndrome by lumbar intrathecal NMDA infusion.

Malmberg and Yaksh (1992) reported that hyperalgesia mediated by spinal glutamate or substance P receptor is blocked by spinal cyclooxygenase inhibition.

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H.1.2.11.6

Chemotherapy-Induced Pain

PURPOSE AND RATIONALE

Within the numerous adverse effects associated with antineoplastic drugs, painful peripheral neuropathy is frequent. Vincristine, an antineoplastic agent widely used in cancer therapy, was found to be neurotoxic for all treated patients and can induce peripheral neuropathy (Windebank 1999). Aley et al. (1996), Authier et al. (1999, 2003) and Marchand et al. (2003) developed an animal model of nociceptive neuropathy using repeated injections of vincristine.

PROCEDURE

Male Sprague Dawley rats weighing 180–200 g received five intravenous injections of 150 μ g/kg vincristine every 2 days until a cumulative dose of 750 μ g/kg was reached. Thresholds to paw pressure were determined before and 14 days after vincristine treatment. Test drug was then applied and the vocalization thresholds were determined 15, 30, 45, 60, 90, and 120 min after this injection.

The C-fiber-evoked flexor reflex elicited in the right hind limb (Falinower et al. 1994; Mestre et al. 1997) was recorded from halothane-anesthetized rats. Rectangular electric pulses of 6–7 mA strength and 2 ms duration were applied every 10 s to the sural nerve receptive field by means of two stainless steel needles inserted into the skin of toes 4 and 5. The C-fiber-evoked reflex response (electromyographic responses) was recorded from the ipsilateral biceps muscle by utilizing another pair of stainless steel needles. Once a stable threshold C reflex response was obtained, the stimulus strength was increased by a factor of threefold. Test drug was then injected in various doses and the mean C-fiber reflex (mean of the 12 C-fiber reflexes recorded during the 2-min period) was calculated every 2 min between 25 and 35 min.

EVALUATION

The data analysis was performed by a two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test for the time course of the effect of drug on mechanical nociceptive thresholds. For the C reflex, results were expressed as mean percentage inhibition of the integrated C reflex responses obtained between 25 and 35 min after drug injection and plotted against log dose, allowing ED₅₀ calculation.

MODIFICATIONS OF THE METHOD

Lynch et al. (2005) tested the effect of a nicotinic acetylcholine agonist against allodynia in rats in the vincristine-induced pain model.

Polomano et al. (2001) described painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel.

Dalziel et al. (2004) described allodynia in rats infected with varicella zoster virus as an animal model for **post-herpetic neuralgia**.

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H.1.2.11.7

Trigeminal Neuropathic Pain Model

PURPOSE AND RATIONALE

Trigeminal neuralgia is an extreme form of neuropathic pain. Although the pathophysiology of the disorder is uncertain, vascular compression of the trigeminal root resulting in damage to primary afferent neurons is thought to play a major role in the generation of pain. Idänpään-Heikkilä and Guilbaud (1999) used a rat model of **trigeminal neuropathic pain**, developed by Gregg (1973), Jacquin and Zeigler (1983), Vos and Maciewicz (1991), Vos et al. (1994), and Vos and Strassman (1995), where the neuropathy is produced by a chronic constriction injury of the infra-orbital branch of the trigeminal nerve, and studied the effects of various drugs on this purely sensory model of neuropathic pain.

PROCEDURE

Male Sprague Dawley rats weighing 175–200 g were used. The head of the rat, which was anesthetized with sodium pentobarbital 50 mg/kg i.p. and treated with 0.4 mg/kg atropine i.p., was fixed in a stereotaxic frame. A mid-line scalp incision was made exposing skull and nasal bone. To expose the intra-orbital part of the left infra-orbital nerve, the edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was dissected free. To give access to the infra-orbital nerve, the orbital contents were gently deflected with a cotton-tipped wooden rod. The infra-orbital nerve was dissected free at its most rostral extent of the orbital cavity, just caudal to the infra-orbital foramen. Two chromic catgut (5–0) ligatures (2 mm apart) were loosely tied around the infra-orbital nerve. The ligatures reduced the diameter of the nerve by just a noticeable amount and retarded, but did not interrupt, the epineurial circulation. The scalp incision was closed with silk sutures.

Before the first actual stimulation session, the rats were allowed to adapt to the observation cage and to the testing environment. During this period, the experimenter reached slowly into the cage to touch the walls with a plastic rod, similar to the ones on which the von Frey filaments were mounted.

For mechanical stimulation, a graded series of ten von Frey filaments with a bending force of between 0.217 and 12.5 g was used. The stimuli were applied within the infra-orbital nerve territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. The complete series of von Frey hair intensities was presented in an ascending series and either a brisk withdrawal of the head or an attack/escape reaction was considered as the mechanical threshold. Local injection of a local anesthetic (articaine) into the rostral orbital cavity of the lesioned side, in close proximity to the ligated infra-orbital nerve, increased the mechanical threshold to the upper level. The duration of the effect was dose-dependent.

EVALUATION

Data are expressed as means \pm SEM. The non-parametric Kruskal–Wallis one-way ANOVA was used and comparisons between the groups were performed using the Mann–Whitney *U*-test.

MODIFICATIONS OF THE METHOD

Christensen et al. (2001) studied the effect of gabapentin and lamotrigine on mechanical allodynia-like behavior in the rat model of trigeminal neuropathic pain.

MODIFICATIONS OF THE METHOD

Deseure et al. (2002) studied the effects of acute i.p. injections of 5-HT_{1A} receptor agonists on mechanical allodynia in a rat model of trigeminal pain.

Cutrer and Moskowitz (1996) studied the actions of valproate and neurosteroids in a **guinea pig model** of trigeminal pain. Hartley guinea pigs were pretreated with valproate or allopregnanolone 30 min prior to activation of trigeminal afferent fibers via intracisternal

activation of trigeminal afferent fibers via intracisternal

injection of capsaicin. The effects were examined on c-fos expression within the trigeminal nucleus caudalis.

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H.1.2.11.8

Migraine Model in Cats

PURPOSE AND RATIONALE

In order to simulate pain experienced by humans in migraine attacks, Storer and Goadsby (1997) developed a model of **craniovascular pain in cats** by stimulating the superior sagittal sinus and monitoring trigeminal neuronal activity using electrophysiological techniques.

PROCEDURE

Adult cats were anesthetized with α -chloralose (60 mg/kg i.p.), paralyzed (gallamine 6 mg/kg i.v.) and ventilated. The superior sagittal sinus was accessed and isolated for electrical stimulation by a mid-line circular craniotomy. The region of the dorsal surface of C₂ spinal cord was exposed by a laminectomy and an electrode placed for recording evoked activity by sinus stimulation and spontaneous activity of the same cells. Signals were amplified and monitored on-line. Cells were recorded that were activated by stimula-

tion of the sinus and were also spontaneously activated. Cells fired with latencies consistent with A δ and C fibers, generally firing three or four times per stimulus (0.3 Hz, 250 μ s duration, 100 V) delivered to the sinus. Both evoked and spontaneous firing could be inhibited by iontophoretic application of serotonin (5-HT)_{1B/1D} agonists.

EVALUATION

The suppression or activation of cell firing was determined from both peri-stimulus and post-stimulus histograms using the criteria of a shift of >30% from baseline. Drug comparisons were made using the Kruskal-Wallis one-way analysis of variance assessing significance at the $P < 0.05$ level.

MODIFICATIONS OF THE METHOD

Using the same method, Goadsby et al. (2002) studied the inhibition of trigeminovascular nociceptive transmission by adenosine A₁ receptor agonists.

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H.1.3

Side Effects of Central Analgesic Drugs

See Chap. I.J.

H.2

Peripheral Analgesic Activity

H.2.0.1

General Considerations

The differentiation between central and peripheral analgesic drugs is nowadays of more or less historical value. Most of the so called peripheral analgesics possess anti-inflammatory properties and in some cases also antipyretic activity besides analgesia. For many of them the mode of action has been elucidated as an inhibition of cyclooxygenase in the prostaglandin pathway. Nevertheless, new peripheral analgesics have to be tested not only for their *in vitro* activity on cyclooxygenase but also for their *in vivo* activity.

The most commonly used methods for measuring peripheral analgesic activity are the writhing tests

in mice (various modifications) and the RANDALL-SELITTO-test in rats.

H.2.0.2

Writhing Tests

PURPOSE AND RATIONALE

Pain is induced by injection of irritants into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior which is called writhing. The test is suitable to detect analgesic activity although some psychoactive agents also show activity. An irritating agent such as phenylquinone or acetic acid is injected intraperitoneally to mice and the stretching reaction is evaluated. The reaction is not specific for the irritant.

PROCEDURE

Mice of either sex with a weight between 20 and 25 g are used. Phenylquinone in a concentration of 0.02% is suspended in a 1% suspension of carboxymethylcellulose. An aliquot of 0.25 ml of this suspension is injected intraperitoneally. Groups of 6 animals are used for controls and treated mice. Preferably, two groups of 6 mice are used as controls. Test animals are administered the drug or the standard at various pretreatment times prior to phenylquinone administration. The mice are placed individually into glass beakers and five min are allowed to elapse. The mice are then observed for a period of ten min and the number of writhes is recorded for each animal. For scoring purposes, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The formula for computing percent inhibition is: average writhes in the control group minus writhes in the drug group divided by writhes in the control group times 100%. The time period with the greatest percent of inhibition is considered the peak time. A dose range is reserved for interesting compounds or those which inhibit writhing more than 70%. Compounds with less than 70% inhibition are considered to have minimal activity.

EVALUATION

A dose range is run in the same fashion as the time response except 8 animals/ group are tested at the peak time of drug activity. Four drug groups and a vehicle control group are employed. Animals are dosed and tested in a randomized manner. An estimated ED_{50} is calculated. Doses of 1.0 mg/kg p.o. indomethacin, 30 mg/kg p.o. acetylsalicylic acid, 40 mg/kg p.o. amidopyrine and 80 mg/kg p.o. phenacetin have been found to be ED_{50} values.

CRITICAL ASSESSMENT OF THE TEST

In this test both central and peripheral analgesics are detected. The test, therefore, has been used by many investigators and can be recommended as a simple screening method. However, it has to be mentioned that other drugs such as clonidine and haloperidol also show a pronounced activity in this test. Because of the lack of specificity, caution is required in interpreting the results, until other tests have been performed. Nevertheless, a good relationship exists between the potencies of analgesics in writhing assays and their clinical potencies.

MODIFICATIONS OF THE METHOD

Instead of a phenylquinone suspension, 0.1 ml of a 0.6% solution of acetic acid is injected intraperitoneally to mice with an weight between 18 and 25 g (Koster et al. 1959; Taber et al. 1969). The response is similar to that after phenylquinone. Some authors have used this method together with observation of changes in capillary permeability in order to distinguish between narcotic and non-narcotic analgesics (Whittle 1964).

Eckhardt et al. (1958), Collier et al. (1968), Loux et al. (1978) showed that several substances are able to elicit the writhing response. For example, Amanuma et al. (1984) as well as Nolan et al. (1990) used as irritant intraperitoneal injections of acetylcholine.

Emele and Shanaman (1963), Burns et al. (1968) proposed bradykinin being an endogenous transmitter of pain as irritant.

Sancillo et al. (1987) induced abdominal constriction in mice by intraperitoneal injection of 31.6 μ g/kg of prostaglandin E_1 .

Bhalla and Bhargava (1980) described a method for assessing aspirin-like activity using aconitine to induce writhing.

Adachi (1994) described a device for automatic measurement of writhing in mice.

Analgesic effects of non-acidic non-steroidal anti-inflammatory drugs in the acetic acid writhing test after intracisternal administration have been found by Nakamura et al. (1986).

The writhing phenomenon can also be observed in **rats** (Fukawa et al. 1980). The writhing responses were induced by intraperitoneal injection of 4% sodium chloride solution. Narcotic and non-narcotic analgesics, antipyretic and nonsteroidal anti-inflammatory drugs were effectively evaluated at relatively low doses. Methamphetamine also showed an analgesic action. VonVoigtlander and Lewis (1982, 1983) induced writhing in rats by injection of

7 ml air or 6% aqueous saline into the peritoneal cavity.

Ethacrinic acid-induced writhing response in rats was used by Björkman et al. (1992).

Schweizer et al. (1988) described a photoelectronic motility monitoring apparatus to measure automatically the writhing movements. A good correlation was found between ED_{50} values after oral administration in mice and the clinically effective oral doses in man.

Heapy et al. (1993) induced the abdominal constriction response in mice by intraperitoneally injecting 0.4 ml of either 0.25% acetic acid, 7.5 mg/ml kaolin suspension, 2.4 mg/ml zymosan solution, or 25 μ g/ml bradykinin solution.

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H.2.0.3

Pain in Inflamed Tissue (RANDALL-SELITTO-Test)

PURPOSE AND RATIONALE

This method for measuring analgesic activity is based on the principle that inflammation increases the sensitivity to pain and that this sensitivity is susceptible to modification by analgesics. Inflammation decreases the pain reaction threshold and this low pain reaction threshold is readily elevated by non-narcotic analgesics of the salicylate-amidopyrine type as well as by the narcotic analgesics. Brewers yeast has been used as an inducer for inflammation which increases pain after pressure.

PROCEDURE

Groups of male Wistar rats (130 to 175 g) are used. Only for oral testing the animals are starved 18 to 24 h prior to administration. Otherwise, the route of administration can be intraperitoneal or subcutaneous. To induce inflammation, 0.1 ml of a 20% suspension of Brewer's yeast in distilled water is injected subcutaneously into the plantar surface of the left hind paw of the rat. Three hours later, pressure is applied through a tip to the plantar surface of the rat's foot at a constant rate by a special apparatus to the point at which the animal struggles, squeals or attempts to bite. The apparatus being used has been modified by various authors such as using the Analgy Meter (Ugo Basile, Apparatus for Biological Research, Milan, Italy). Each animal is tested for its control pain threshold. Any animal with a control pain threshold greater than 80 g is eliminated and replaced.

For a time response, groups of at least 7 animals are used, four groups for the agent to be tested and one for the vehicle control. The tests are done at 15 min intervals after subcutaneous administration and at 30 min intervals after oral administration for any change in pain threshold. The interval of time which indicates the greatest increase in pain threshold is regarded as the peak time.

A dose range is obtained in the same manner as the time response. The drug to be tested is administered in a randomized manner. The pain threshold is recorded at time zero and again at the determined peak time.

EVALUATION

The mean applied force is determined for each time interval tested. The percentage increase in pain threshold is calculated by subtracting the applied force of the vehicle control from the applied force of the drug group which is divided by the applied force of the vehicle control in order to give the percentage of increase in pain threshold of the drug group. Doses of 50 mg/kg s.c. Na salicylate, 50 mg/kg amidopyrine, 3 mg/kg s.c. morphine, 12.5 mg/kg s.c. codeine or pethidine have been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The method originally described by RANDALL and SELITTO has been used by many investigators and has been proven to detect central analgesics as well as peripheral analgesics. Peripherally acting analgesics such as the nonsteroidal anti-inflammatory drugs increase only the threshold of the inflamed paw, whereas opiate analgesics increase also the threshold of the intact paw (Dubinsky et al. 1987). In most modifica-

tions, the assay has a shallow dose-response curve. Nevertheless, the ED_{50} values of nonsteroidal anti-inflammatory drugs in this test showed a good correlation with human doses (Romer 1980).

MODIFICATIONS OF THE METHOD

The test has been modified by various authors. In some instances the pressure on the inflamed paw has been omitted. Instead the animals were allowed to walk on a metal grid. The gait of the animals is assessed by an observer using a scoring system:

- 0 = three-legged gait
- 0.5 = marked limping
- 1 = normal gait.

The scores are transformed into percent analgesia.

Other noxious stimuli were used to induce inflammation and hyperalgesia, such as carrageenin (Winter et al. 1962), Freund's adjuvant or prostaglandin E_2 (Ferreira et al. 1978a).

Vinegar et al. (1990) injected 0.1 ml of 0.25% solution of trypsin into the subplantar region and applied the load force 60 min later. They found a biphasic hyperalgesia and relatively low ED_{50} values for central and peripheral analgesics.

Technically, the method has been improved by several authors, such as Takesue et al. (1969).

Chipkin et al. (1983) modified the test by decreasing the rate of acceleration of the noxious stimulus (mechanical pressure) on the inflamed paw from 20 to 12.5 mm Hg/s and an extension of the cut-off time from 15 to 60 s. This modification is claimed to discriminate analgesics active against mild to severe clinical pain (narcotic-like) from those only useful against mild to moderate pain (non-narcotic-like).

Randall-Selitto analgesy meters are commercially available (e.g., IITC Life Science, Woodland Hills, CA, USA).

Central and peripheral analgesic action of aspirin-like drugs has been studied with a modification of the Randall-Selitto method applying constant pressure to the rat's paw by Ferreira et al. (1978b).

A modification of an analgesia meter for paw pressure antinociceptive testing in neonatal rats was described by Kitchen (1984).

Learning and retention has been tested in rats by Greindl and Preat (1976) inducing pain by a light quantifiable pressure applied to the normal hind paw.

Hargreaves et al. (1988) described a sensitive method for measuring thermal nociception in cutaneous hyperalgesia in rats. One paw was injected with

0.1 ml carrageenan solution, the other paw with saline. The rats were placed in chambers with glass floor and radiant heat was directed to the paws. A photoelectric cell detected the light reflected from the paw and turned off the radiant heat when paw movement interrupted the reflected light.

Perkins et al. (1993) described hyperalgesia after injection of 100 μ l of Freund's adjuvant into the knee of anesthetized rats. After 64–70 h the animal was placed with each hind paw on a pressure transducer and a downward force was exerted until the uninjected leg was bearing 100 g. At this point animals were less tolerant to a load on the injected leg, indicating a hyperalgesic response.

Davis et al. (1996) induced mechanical hyperalgesia by injection of substance P and capsaicin in the rat knee joint and measured the download force tolerated by the injected leg.

Ferreira et al. (1993a, b) induced hyperalgesia by intraplantar injection in the hindpaw of rats of various agents, e. g., bradykinin, carrageenin, LPS, PGE₂, dopamine, TNF α , IL-1 β , IL-6 and IL-8. A constant pressure of 20 mmHg was applied to the hind paws and discontinued when the rats presented a typical freezing reaction.

Subplantar injection of 0.1 μ g of serotonin in the rat results in a brief period (up to 20 min) of increased pain sensitivity to an applied force (hyperalgesia) which precedes a longer period of decreased pain sensitivity (hypoalgesia). Vinegar et al. (1989) used this phenomenon for pharmacologic characterization of the analgesic response.

Similarly, a biphasic algesic behavior after subplantar injection of 250 μ g of trypsin was described by Vinegar et al. (1990)

Courteix et al. (1994) proposed the Randall-Selitto paw pressure test in rats with streptozocin-induced diabetes as a model of chronic pain with signs of hyperalgesia and allodynia that may reflect signs observed in diabetic humans.

Amann et al. (1955, 1996) evaluated local edema and effects on thermal nociceptive threshold after intraplantar injection of nerve growth factor into the rat hind paw and studied the effect of a 5-lipoxygenase inhibitor in this test.

Zhou et al. (1996) tested the effects of peripheral administration of NMDA, AMPA or KA on pain behavior in rats. A 28-gauge needle was inserted in the skin of rats proximal to the footpads and advanced about 1 cm so that the tip reached the base of the third toe. A bolus of 20 μ l containing concentrations between 1 and 10,000 μ M of KA, NMDA or AMPA. For behav-

ioral testing, each animal was placed in a Plexiglas box on a wire mesh screen. Mechanical stimuli were applied using four von Frey filaments with different bending forces. Each von Frey filament was applied 10 times to the skin on the base of the third toe. The paw withdrawal was rated as scores allowing dose-response curves for the hyperalgesic effects of excitatory amino acids. Furthermore, using the highest concentration of the stimulant, effects of antagonists were tested.

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H.2.0.4

Mechanical Visceral Pain Model in the Rat

PURPOSE AND RATIONALE

Animal models designed to test the effectiveness of analgesic agents against visceral pain typically rely on noxious chemical irritation of the peritoneum, e. g., acetic acid and phenylquinone induced writhing tests based on acute inflammation. Ethical constraints prevent repeated assessments in a single animal, thereby compounding the difficulty of assessing development of tolerance to analgesic agents. To overcome these constraints, a model for mechanical visceral pain was developed based on repeatable and reversible distension of duodenum in the rat (Coburn et al. 1989; deLeo et al. 1989).

PROCEDURE

A one-piece balloon catheter is prepared composed of PE 50 tubing with a terminal latex rubber balloon which is 7.5 mm long and distensible to hold more than 1.5 ml fluid. Male Sprague Dawley rats weighing 175–200 g are anesthetized with N₂O and halothane. The abdomen is shaved and a 2.5 cm incision is made transversely just below the left costal margin. On the greater curvature of the stomach an incision is made 10 to 20 mm above the pylorus

and a purse string is accomplished with 4–0 silk prior to gastrotomy. Through a 2 mm gastrotomy the catheter is introduced and advanced through the pylorus to the first portion of the duodenum (approximately 15–20 mm from the pylorus). The purse string is tied snugly closing the gastrotomy around the catheter. The catheter is tunneled to the base of the scull, externalized and anchored to the dermis with a silicon sleeve and suture. The animals recover from anesthesia within 5 min. Following a 4–5 days recovery period, the duodenal distension volume is determined by the mean threshold that produces writhing (usually 0.5 to 0.7 ml). For the test, the animals are randomized and administered either saline, the standard (0.1, 0.25, 1, and 10 mg/kg indomethacin i.p.) or the test drug in various doses prior to challenging. The animals are placed in a polypropylene box and challenged by inflating the balloon with saline, using a 1 ml calibrated syringe, pulsed 5 times over 30 s and then distended for 1 min. Behavioral responses are scored:

- 0 = Normal behavior defined as exploration, escape attempts and resting
- 1 = Slightly modified behavior defined as cessation of exploration, focusing, wet-dog shake, excessive facial grooming, teeth chattering and deep breathing
- 2 = Mildly to moderately modified behavior defined as retching-like activity, hunching, abdominal grooming or nipping and immobility of the hind limbs (disappears with removal of the stimulus).
- 3 = Severely modified behavior defined as stretching of the hindlimbs, arching and dorsoflexion of the hind paws.
- 4 = Intensive visceromotor activity defined as repetitive stretching of the body, extension of the hind limbs, and pelvis, frequent rotating sideward, i. e., writhing.

EVALUATION

The average scores of the groups are plotted on semi log paper and *ED*₅₀ values are determined by best line fit.

CRITICAL ASSESSMENT OF THE METHOD

In the mechanical visceral pain model in the rat, morphine and indomethacin have been found to be active but not other agents involved in prostaglandin inhibition, like acetylsalicylic acid and mefenamic acid. Other mechanisms besides those involving the arachidonic acid cascade have to be investigated.

MODIFICATIONS OF THE METHOD

Ness and Gebhart (1988) used colorectal distension as a noxious visceral stimulus in awake, unanesthetized, unrestrained rats. A 7–8 cm flexible latex balloon was inserted intra-anally under ether anesthesia and kept in position by taping to the base of the tail. Opening a solenoid gate to a constant pressure air reservoir initiated a 20 s, constant pressure stimulus in the descending colon and rectum. Femoral arterial and venous catheters were tunneled subcutaneously and exteriorized at the back of the neck. Teflon-coated stain-less steel wire electrodes were stitched into the external oblique musculature immediately superior to the inguinal ligament for electromyographic recordings. Blood pressure and heart frequency increase were proportional to the degree of colorectal distension. These effects could be dose-dependently antagonized by morphine and clonidine.

Renal pelvis distension with a pressure of 80 cm H₂O causes a decline in mean arterial blood pressure in pentobarbital-anesthetized rats. Brasch and Zetler (1982) used this blood pressure response, which disappears rapidly after cessation of the distension, to study the effects of analgesic drugs known to be effective in renal colic pain in man.

Moss and Sanger (1990) measured falls in diastolic blood pressure and intragastric pressure after distension of the duodenum by rapid application of intraluminal pressure (10–75 cm H₂O) in anesthetized rats. The distension-induced responses were blocked by pretreatment with morphine, an action reversible by injection of naloxone. Bilateral cervical vagotomy reduced the distension-evoked fall in intragastric pressure but had no effect on the corresponding fall in blood pressure.

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H.2.0.5**Antagonism Against Local Effects of Bradykinin****PURPOSE AND RATIONALE**

Guzman et al. (1962) and Lim et al. (1964) described the responses (vocalization, respiratory and blood pressure changes) to intra-arterial injection of bradykinin and other algescic agents in cats and dogs. Deffenu et al. (1966) and Blane (1968) used the bradykinin-induced effects after intra-arterial injection in rats as an assay for analgesic drugs. Paravascular sensory nerves which accompany blood vessels throughout the body to end in unmyelinated free-branching terminals close to the capillaries and venules most likely carry the chemoreceptors of pain (Lim 1970). Due to rapid enzymatic degradation, bradykinin is ineffective as noxious stimulus after intravenous or oral administration.

PROCEDURE

Male Wistar rats weighing 280–320 g are lightly anesthetized with ether. A polyethylene catheter with an internal diameter of 0.5 mm is inserted centripetally into the right carotid artery. The catheter is passed through the subcutaneous tissues to protrude from the back of the animal. One hour after recovery from anesthesia, the first dose of bradykinin is injected into the catheter producing dextro-rotation of the head, flexing of the forelimb and occasionally squeaking. For each rat the minimum dose of bradykinin is determined necessary to provoke these effects. The test compounds are applied subcutaneously or intraperitoneally 15 min prior to injection of the threshold dose of bradykinin. The bradykinin injections are repeated in 5 min intervals until the bradykinin effect reappears. Each rat receives one drug at one dose level.

EVALUATION

The criterion for protection is the disappearance of the bradykinin effect after at least 2 consecutive doses of bradykinin. Using groups of 10 rats for various dose levels, *ED*₅₀ values are calculated.

CRITICAL ASSESSMENT OF THE METHOD

Not only narcotic analgesics, but also pyrazolones and phenacetin or acetylsalicylic acid are active in this test. In some animals, the bradykinin-induced response can be diminished after repeated injections, classified as the noxious-adaptable group (Satoh et al. 1979).

MODIFICATIONS OF THE METHOD

Haubrich et al. (1990) tested analgesic activity by the intracarotid bradykinin-induced head/forepaw flexion in the rat. Male Charles River rats (280–320 g) fasted overnight were prepared surgically under light ether anesthesia by insertion of a capped polyethylene cannula (PE-60) centripetally into the right carotid artery and then exteriorizing the cannula to a harness on the back, to permit repeated i.a. injections. The rats were allowed to recover at least 2 h from the surgery, and then given single i.a. injections of bradykinin (triacetate salt in 0.2 ml 0.9% NaCl per injection) at 10-min intervals to determine the threshold dose which produced marked dextrorotation of the head and flexion of the right forepaw of each rat. This response was elicited by threshold doses of bradykinin ranging from 0.1 to 0.5 µg/injection. After administration of the test drugs, the response of the threshold dose of bradykinin was determined at 10-min intervals for 1 h and then at 20-min intervals during the second hour. ED_{50} values were determined by probit analysis of the maximum percentage of rats that failed to respond to bradykinin at each dose of test drug any time within the 2-h test period.

Collier and Lee (1963) described nociceptive responses of **guinea pigs** to intradermal injections of bradykinin and kallidin-10.

Vargaftig (1966) measured the effect of non-narcotic analgesics on the hypotension induced by intra-arterial injection of bradykinin in **rabbits**.

Adachi and Ishii (1979) used the response to injection of bradykinin into the femoral artery of guinea pigs for quantitative assessment of analgesic agents.

Griesbacher and Lembeck (1987) and Lembeck et al. (1991) used the reflex hypotensive response as an indicator of nociception after injection of bradykinin into the ear artery of anesthetized rabbits. Rabbits were anesthetized and the blood pressure was recorded from the carotid artery. The central artery of one ear was cannulated and the ear was separated from the head with the exception of the auricular nerve, which remained connected to the head. The ear was perfused with Tyrode solution to which acetylcholine and bradykinin were added. The reflex fall in blood pressure induced by bradykinin and acetylcholine were monitored. The effect could be inhibited by bradykinin antagonists.

Heapy et al. (1993) tested the effects of the bradykinin antagonist HOE140 on the abdominal constriction response after intraperitoneal injection of bradykinin to **mice**.

Further Methods Used to Study the Role of Bradykinin and Bradykinin Antagonists in Inflammation and Algesia

Teixeira et al. (1993) investigated the mechanisms of inflammatory response induced by extracts of *Schistosoma mansoni* larvae in guinea pig skin. *Biomphalaria glabrata* snails with patent *Schistosoma mansoni* infections were induced to shed cercariae by exposure to light and water with a temperature of 31°C. The cercariae were concentrated, homogenized and extracts prepared. Purified eosinophils or neutrophils obtained from peritoneal exudates were radiolabeled by incubation with ^{111}In chelated to 2-mercaptopyridine-*N*-oxide. Radiolabeled leukocyte infiltration and edema formation were measured simultaneously at injected skin sites. [^{125}I]Human serum albumin was added to the labeled leukocytes and these were injected i.v. into anesthetized guinea pigs. After 15 min the extracts of cercariae were locally injected with/or without the inhibitors. After 2 h, the animals were sacrificed and the injected sites were punched out with a 17-mm punch. Serum exudation and leukocyte infiltration were measured by counting the two isotopes. The bradykinin antagonist HOE 140 reduced substantially the extract-induced inflammation.

Ahluwalia et al. (1994) induced plasma protein extravasation in the rat urinary bladder by i.p. injection of cyclophosphamide mediated by capsaicin-sensitive primary afferent neurons which could be significantly inhibited by the bradykinin B_2 receptor antagonist HOE140 and the tachykinin NK_1 receptor antagonist RP67,580.

Davis and Perkins (1994a, b) described a model of persistent inflammatory mechanical hyperalgesia using intra-articular injections of bradykinin or cytokines into the knee joint of rats.

Lecci et al. (1995) analyzed the local and reflex responses to bradykinin on rat urinary bladder motility *in vivo*.

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and central level have been discussed. Schaible and Schmidt (1983a, b, 1985, 1987, 1988) performed electrophysiological experiments in anesthetized cats and rats after local mechanical stimulation and after induction of acute arthritis of the knee joint. With this method Xe et al. (1990a, b), Neugebauer et al. (1994) found evidence for a spinal antinociceptive action of antipyretic analgesics, such as dipyron.

PROCEDURE

In cats weighing 2.0–4.0 kg anesthesia is induced by i.m. injection of 15–30 mg/kg ketamine hydrochloride followed by i.v. injection of 60 mg/kg α -chloralose. After immobilization with i.v. pancuronium bromide the cats are artificially ventilated. The skin of the right thigh is incised from rostral of the inguinal fossa to a point below the medial condyle of the tibia. The tendon of the sartorius muscle is cut close to its insertion at the capsule of the knee joint. The muscle is removed to expose the medial aspects of the joint and the medial articular nerve (MAN). The thigh is rigidly fixed to the mounting table by a threaded bolt fitted through the femur so that the lower leg can be flexed and extended in the horizontal plane. The saphenous nerve is cut in the inguinal fossa for recording. Bipolar electrodes are inserted at the MAN near the knee for en passant stimulation of articular afferents. **Extracellular recordings from single MAN units** in the saphenous nerve are performed using platinum wire electrodes. According to their conduction velocities units are classed as group IV afferents (<2.5 m/s, unmyelinated axons) or group III afferents (2.5–20 m/s, thinly myelinated axons).

For **recordings from spinal cord neurons** the spinal segments T12–L7 are exposed by laminectomy. The spinal cord is transected at the lower thoracic region after injection of 0.1 ml of 1% procaine hydrochloride solution to prevent mechanical activation of axons in the long spinal tracts. The animals are fixed to a rigid frame with spinal and pelvic clamps. A pool is formed by skin flaps and filled with warm paraffin oil. The upper lumbar spinal cord is mounted on a pair of platinum wire stimulating electrodes surrounding the whole cord. Ascending tract neurons are identified by electrical stimulation (Neugebauer and Schaible 1990). Single spinal neurons that can be excited by mechanical stimulation of the knee joint tissue are recorded extracellularly using glass-insulated carbon filament electrodes. The neurons are either nociceptive specific neurons responding only to noxious mechanical stimuli or wide dynamic range neurons re-

H.2.0.6

Effect of Analgesics on Spinal Neurons

PURPOSE AND RATIONALE

The mode of action of peripheral analgesics is still a matter of debate. Besides inhibition of the arachidonic acid derived pathway, activities on the spinal

sponding to innocuous stimuli but showing strongest responses to stimuli of noxious intensity.

Acute arthritis in the right knee joint is induced several hours before recordings are started by injecting 0.3–0.5 ml of 4% kaolin suspension and 15–20 min later 0.3 ml of 2% carrageenan solution. Acute arthritis develops within 1–3 h.

Action potentials are displayed on a storage oscilloscope, amplified, filtered, fed to a window discriminator and processed using an interface and a personal computer for construction of peristimulus time histograms. After a control period of at least 40 min during which a stable discharge rate of the afferent or spinal cord unit is obtained, the test substances are administered i.v. in various doses. Effects of the test substance on ongoing and mechanically evoked activity (by movements, pressure stimuli) are determined.

EVALUATION

Ongoing activity is counted every min. The means and standard deviations in 10 min periods are calculated before and after drug application. The values after drug injection are calculated as percentage of control values. To calculate the net effects of the different mechanical stimuli, the number of impulses in the preceding 30 s is subtracted from the total discharges during the stimulus. The responses to at least 4 stimuli before drug application are averaged and set to 100%. The responses to the different mechanical stimuli after drug administration are expressed as a percentage of the controls. Statistical significance is evaluated using the *t*-test for unpaired samples.

MODIFICATIONS OF THE METHOD

Using the model of kaolin-induced arthritis in the knee of rats, Han and Neugebauer (2005) and Han et al. (2005) developed a computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior.

Several other electrophysiological methods have been applied to elucidate the mode of action of non-opioid analgesic agents. Carlsson et al. (1986, 1988), Jurna and Brune (1990) recorded the activity from single neurons in the dorsomedial part of the ventral nucleus of the thalamus in rats. Activity was elicited by supramaximal stimulation of nociceptive afferents in the sural nerve. In addition, activity was recorded in ascending axons of the spinal cord.

Chapman and Dickenson (1992) studied the spinal and peripheral roles of bradykinin and prostaglandins in nociceptive processing in the rat by recording C-

fibres activity in the dorsal spinal horn after injection of formalin into the center of the respective field of the toe of the hind paw.

Dray et al. (1992) described a preparation of the neonatal rat spinal cord with functionally connected tail maintained *in vitro*. The preparation was placed in a chamber and the spinal-cord and tail were separately superfused with a physiological salt solution. Peripheral nociceptive fibers were activated by superfusion of the tail with bradykinin, capsaicin or by a superfusate heated to 48–50°C (noxious heat). The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root.

Malmberg and Yaksh (1992) described a direct analgesic action of NSAIDs through spinal cyclooxygenase inhibition by blocking the thermal hyperalgesia in rats induced after intrathecal administration of excitatory amino acids or substance P.

A simple technique for intrathecal injections by lumbar puncture in unanesthetized mice was described by Hylden and Wilcox (1980).

Mestre et al. (1994) described a method for performing direct intrathecal injections in rats without introducing a spinal catheter.

Bahar et al. (1984) performed chronic implantations of nylon catheters into the subarachnoid space of Wistar rats and marmosets and tested the effects of local anesthetics.

McQueen et al. (1991) investigated the effects of paracetamol and lysine acetylsalicylate on high-threshold mechano-nociceptors by recording neural activity from the inflamed ankle joint in anesthetized rats with mild adjuvant-induced mono-arthritis.

Yamamoto and Yaksh (1992) studied the effects of excitatory amino acid antagonists administered through chronically implanted lumbar intrathecal catheters on the thermal hyperesthetic state induced by unilateral partial ligation of the sciatic nerve in rats.

Hashimoto and Fukuda (1990) described a spinal cord injury model produced by spinal cord compression in the rat.

Aanonsen and Wilcox (1987) tested effects of spinally administered opioids, phencyclidine and sigma agonists on the action of intrathecally administered NMDA in the tail-flick, hot-plate and biting and scratching nociceptive tests in **mice**.

Brambilla et al. (1996) demonstrated that intrathecal administration of AMPA produced a dose-dependent behavioral syndrome in mice characterized by caudally directed biting, which could be antagonized by peripheral administration of AMPA-receptor and NMDA-receptor antagonists.

Aanonsen et al. (1990) tested the effect of iontophoretically applied excitatory amino acid agonists, such as NMDA, AMPA, quisqualate and kainate, on the firing rate of rat spinal neurons after peripheral noxious stimulation.

Cumberbatch et al. (1994) studied the roles of receptors for AMPA in spinal nociceptive and non-nociceptive transmission on dorsal horn wide dynamic range neurones in anesthetized spinalized rats. The effects of systemically administered competitive and non-competitive AMPA antagonists were examined on responses to peripheral noxious heat and non-noxious tap stimuli as well as to iontophoretic AMPA and NMDA.

With this technique, Chizh et al. (1994) studied the effects of intravenous administration of AMPA antagonists to iontophoretically applied excitatory amino acids.

Watkins et al. (1994) induced hyperalgesia in rats by intraperitoneally administered lipopolysaccharides as measured by radiation heat tail flick in rats. Intrathecal catheters were implanted into the subdural space surrounding the spinal cord to test the involvement of excitatory amino acids, substance P, CCK and opioids assessing the effects of antagonists.

Mjelle et al. (1993) produced a behavioural syndrome of caudally directed biting in mice by intrathecal injection of either NMDA or AMPA.

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H.2.0.7

Antagonism to Nerve Growth Factor

H.2.0.7.1

General Considerations on Nerve Growth Factor

Nerve growth factor (NGF) is a member of the neurotrophin family of structurally related secreted proteins that includes brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4. Mature neurotrophins are homodimers that are derived by proteolytic cleavage from precursor proteins encoded by separate genes. They bind to two types of receptor: a common receptor, p75^{NTR}, which binds all neurotrophins with a similar affinity; and members of the trk family of receptor tyrosine kinases, trkA, trkB and trkC, which bind different neurotrophins.

NGF and BDNF have a crucial role in the generation of pain and hyperalgesia in several acute and chronic pain states (Woolf et al. 1994; McMahon 1996; Mannion et al. 1999; Thompson et al. 1999; Pezet et al. 2002; Allen and Dawbarn 2006). The expression of NGF is high in injured and inflamed tissues, and activation of the receptor tyrosine kinase trkA in nociceptive neurons triggers and potentiates pain signaling by multiple mechanisms (McMahon et al. 1995; Huang and Reichardt 2003; Hefti et al. 2006) (see also F.2.0.10).

An effective pain therapeutic needs to prevent the activation of trkA by NGF. This may be achieved by agents that remove free NGF, by molecules that prevent binding to trkA, and by molecules that prevent activation of trkA.

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H.2.0.7.2

In Vitro Assays of Nerve Growth Factor

PURPOSE AND RATIONALE

The neurotrophin nerve growth factor (NGF) binds to two receptor types: the tyrosine kinase receptor TrkA and the common neurotrophin receptor p75^{NTR}. Although many of the biological effects of NGF (such as neuronal growth and survival) are associated with TrkA activation, p75^{NTR} also contributes to these activities by enhancing the action of TrkA when receptors are coexpressed (Verdi et al. 1994; Kaplan and Miller 1997). Colquhoun et al. (2004) studied the NGF antagonist PD90780 (Spiegel et al. 1995), which interacts with NGF, preventing its binding to p75^{NTR}. In this study, the actions of this compound were further explored, and it was found that PD90780 is not able to inhibit the binding of either brain-derived neurotrophic factor or neurotrophin-3 to p75^{NTR}, consistent with the direct interactions of the antagonist with NGF. In addition, it was demonstrated that the ability of PD90780 to inhibit NGF-p75^{NTR} interactions is lower when receptors are coexpressed, compared with when p75^{NTR} is the only neurotrophin receptor expressed.

PROCEDURE

Radiolabeled Neurotrophin and Receptor Preparation

The iodination of NGF (mouse 2.5s; Cedarlane Labs, Toronto, ON) and rhBDNF (Alomone Labs, Jerusalem, Israel) was performed as described by Sutter et al. (1979) with modification (Ross et al. 1997). The ability of PD90780 to block neurotrophin binding to the p75^{NTR} receptor was evaluated under various receptor conditions. This was accomplished with

the use of PC12 cells (TrkA and p75^{NTR}), PC12^{nmr5} cells (p75^{NTR} only), and truncated p75^{NTR}. The two cell types were cultured in RPMI 1640 medium with 10% fetal calf serum. Recovery of the cells was permitted with the replacement of the medium with calcium/magnesium-free balanced salt solution followed by a 15-min incubation at 37°C. Cells were centrifuged, and pellets were suspended in HKR buffer (10 mM HEPES, pH 7.35 containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1 g/l glucose, and 1 g/l bovine serum albumin). In the case of truncated p75^{NTR}, the culture medium used to grow PC12 cells was removed and centrifuged to ensure it was free of cells. This medium contained p75^{NTR} extracellular domains previously sloughed by the cells (molecular weight approximately 50 kDa) (DiStefano and Johnson 1988).

Chemical Cross-Linking of ¹²⁵I-NGF to TrkA and/or p75^{NTR} in the Presence of Antagonists and Immunoprecipitation

¹²⁵I-NGF (0.1 nM) alone or in combination with NGF (100 nM), BDNF (10 nM), or PD90780 (100 μM) was incubated with PC12 cells at a concentration of 10⁶ cells/ml in HKR buffer in a volume of 1 ml for 2 h at 4°C with rocking. After binding, 20 μl of the cross-linker bis-(sulfosuccinimidyl)suberate (BS³) was added (final concentration of 0.4 mM) to each sample and incubated at room temperature for 30 min. The cells were washed three times with TBS, after which reducing SDS sample buffer was added to the pelleted cells to dissolve the proteins, or in the case of immunoprecipitations prepared as described below. Cell samples undergoing immunoprecipitations for TrkA or p75^{NTR} were solubilized in lysis buffer (TBS containing 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin) and incubated for 40 min at 4°C. After centrifugation, the lysates were removed to a new tube, and either rabbit polyclonal anti-Trk cytoplasmic domain antibody or rabbit polyclonal anti-p75^{NTR} antibody (9992) (antisera against glutathione S-transferase-fusion protein containing the cytoplasmic domain of p75^{NTR}) was added to the soluble proteins to isolate the respective receptors. The samples were left to incubate at 4°C overnight. Antibody complexes were removed through application and incubation with 70 μl of a 50% slurry of immobilized Protein G (Pierce Chemical, Rockford, Ill., USA) for 2 h at 4°C. The solid phase was washed with lysis buffer three times, with distilled water once, and then the proteins were dissolved in SDS sample buffer. Pro-

teins from cross-linking and immunoprecipitation experiments were separated via 6% SDS-polyacrylamide gel electrophoresis (PAGE).

Chemical Cross-Linking of ¹²⁵I-NGF to p75^{NTR} and Concentration Effect Assays

¹²⁵I-NGF was incubated at 4°C for 2 h with or without PD90780. PC12 or PC12^{nmr5} cells were added at 10⁶ cells/ml, and samples were incubated at 4°C for 2 h with rocking. Bound ¹²⁵I-NGF and p75^{NTR} proteins were cross-linked with final concentrations of 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 2 mM sulfo-*N*-hydroxy-sulfosuccinimide (SNHS) (20 μl of each) and incubated with rocking at room temperature for 30 min. Samples were washed with TBS [10 mM Tris(hydroxymethyl)-aminomethane, pH 8.0, and 150 mM NaCl] three times before the addition of reducing SDS sample buffer to dissolve the proteins. The proteins were then separated on a 6% SDS-PAGE gel. In the experiments involving truncated p75^{NTR}, ¹²⁵I-NGF or ¹²⁵I-BDNF was exposed to the same concentrations of PD90780 and incubated with medium containing truncated p75^{NTR} before cross-linking with EDC/SNHS. The reaction was quenched by adding 15 μl of 1 M glycine followed by 10 min of mixing. The samples were then immunoprecipitated using 192 IgG, which recognizes the extracellular domain of p75^{NTR} (Calbiochem, San Diego, Calif., USA).

Neurotrophin Receptor Binding

Neurotrophins NGF, BDNF, and NT-3 were iodinated, and PC12 and PC12^{nmr5} cells were cultivated and recovered as previously described. Tubes were set up containing single data points that held iodinated neurotrophin (0.5 nM), PD90780 (10 μM), a final concentration of 10⁶ cells/ml and NGF (at 50 nM for non-specific binding) as required, and were then incubated at 4°C for 2 h. Aliquots (100 μl) were layered on top of 200 μl of 10% glycerol in HKR buffer in 0.4-ml tubes. Samples were then centrifuged at 5000 rpm for 2 min, after which the tip containing the cell pellet was cut off and radioactivity present was determined.

TrkA Phosphorylation Assay

Modification of the methods described permitted determination of TrkA phosphorylation (Ross et al. 1998). NGF (40 pM) was incubated with varying concentrations of PD90780 (3, 30, or 300 μM) for 2 h in HKR buffer. PC12 cells used at 10⁶ cells/ml were incubated with NGF and PD90780 solutions for 15 min at 37°C. Samples were washed once with cold PBS and once with cold TBS and then lysed with solutions

containing 500 μM orthovanadate and immunoprecipitated with anti-Trk antibody as previously described. An SDS-PAGE run on 6% gel followed by Western blot analysis performed with antiphosphotyrosine antibody (4G10; UBI, Lake Placid, N.Y., USA) and visualized with ECL (Amersham) permitted the resolution of isolated phosphoproteins. The resulting bands were quantified via densitometry analysis.

NGF Protomer Cross-Linking

^{125}I -NGF (0.1 nM) was incubated for 2 h at 4°C with ZnCl_2 (100 μM), PD90780 (30 μM), or ZnCl_2 and PD90780 (100 and 30 μM , respectively), along with HKR buffer, for a total volume of 0.1 ml. After incubation, BS^3 was added (final concentration of 0.4 mM) in 5 μl volume and set at room temperature for 30 min. Proteins were dissolved with the addition of 50 μl of SDS sample buffer and heating to 95°C for 10 min. Separation of ^{125}I -NGF dimers and ^{125}I -NGF monomers was completed using a 15% acrylamide gel SDS-PAGE.

EVALUATION

Following SDS-PAGE, gels were fixed and dried, and the radio-iodinated ligands cross-linked to receptors were detected via autoradiography. Receptor ligand bands within SDS-PAGE gels were excised, and radioactivity within each band was detected with a Beckman gamma counter. The concentration–effect curves, SEM, IC_{50} values, and 95% confidence intervals (CIs) described in the concentration–effect studies were determined by non-linear regression analyses and carried out by the program GraphPad Prism, version 3.00 (GraphPad Software, San Diego, Calif., USA).

MODIFICATIONS OF THE METHOD

Debeir et al. (1999) described a nerve growth factor mimetic TrkA antagonist that causes withdrawal of cortical cholinergic boutons in the adult rat. A small peptide, C(92–96), which blocks NGF–TrkA interactions, was delivered stereotactically into the rat cortex over a 2-week period, and its effect and potency were compared with those of an anti-NGF monoclonal antibody (mAb NGF30). Two presynaptic antigenic sites were studied by immunoreactivity, and the number of presynaptic sites was counted by using an image analysis system. Synaptophysin was used as a marker for overall cortical synapses, and the vesicular acetylcholine transporter was used as a marker for cortical cholinergic presynaptic sites.

Owolabi et al. (1999) characterized the antiallo-dynamic actions of ALE-0540, a novel nerve growth fac-

tor receptor antagonist that inhibits the binding of NGF to tyrosine kinase (Trk) A or both p75 and TrkA (IC_{50} $5.88 \pm 1.87 \mu\text{M}$, $3.72 \pm 1.3 \mu\text{M}$, respectively), as well as signal transduction and the biological responses mediated by TrkA receptors.

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H.2.0.7.3

In Vivo Assays of Nerve Growth Factor Antagonism

PURPOSE AND RATIONALE

Many studies indicate the role of NGF in pain perception.

Herzberg et al. (1997) reported NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve.

Ma and Woolf (1997) reported that the progressive tactile hyperalgesia induced by peripheral inflammation is nerve growth factor dependent. An i.p. injection

of anti-NGF antiserum (5 μ l/g) 1 h before induction of inflammation by intraplantar complete Freund's adjuvant (CFA) injection and 24 h after reduced the basal inflammatory hypersensitivity and significantly attenuated the progressive increase of spontaneous activity, touch-, pinch- and A β -afferent-evoked responses, as well as the progressive reduction of the mechanical threshold of biceps femoris/semiotendinosus alpha motoneurons normally evoked by repeated (every 5 min) tactile stimulation of the inflamed hindpaw, in decerebrate-spinal rats.

Ro et al. (1999) described the effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve.

Theodosiou et al. (1999) studied the role of nerve growth factor in hyperalgesia due to nerve damage.

Gwak et al. (2003) found attenuation of mechanical hyperalgesia following spinal cord injury by administration of antibodies to nerve growth factor in the rat.

PROCEDURE

Adult Sprague Dawley rats (200–250 g) were spinally hemisected at T13 (Christensen et al. 1996). Under enflurane anesthesia (induction 3% and maintenance 2%) the T11–12 laminae were determined by counting the dorsal spinous processes from the sacrum. The surgical field was then shaved, a longitudinal incision made exposing several segments, and a laminectomy performed at two vertebral segments, T11–T12. The spinal cord was hemisected just cranial to the L1 dorsal root entry zone with a micro-dissecting knife without damaging the major dorsal vessel or vascular branches. An insulin syringe with a 28-gauge needle was placed dorsal-ventrally at the midline of the cord, and pulled laterally to ensure the completeness of the hemisection. The incised skin was sutured, and postoperative care was done. After the hemisection, animals were either treated once a day for 10 days with anti-NGF (anti-nerve growth factor-2.5S, Sigma, i.p., 2 μ g, 0.2 ml), or with saline (0.2 ml), or were untreated.

In the behavioral experiments, rats were housed in clear plastic boxes (8 \times 8 \times 24 cm) above a metal mesh (0.5 \times 0.5 cm) and acclimatized for 30 min to avoid the stress associated with environmental change. Mechanical paw withdrawal threshold to the application of a von Frey filament was measured by using the up-down testing paradigm (Ro et al. 1999). An ascending series of von Frey filaments of incremental force (0.35, 0.53, 0.78, 2.5, 3.7, 5.2, 6.0, and 12.5 g) was applied for 3 s to the middle of the plantar surface of the hind paw, starting with the 2.5 g stimulus (Chaplan et al. 1994).

For electrophysiology experiments, rats were anesthetized with sodium pentobarbital (40–50 mg/kg), with supplementary pancuronium bromide (2–4 mg/kg per h), and artificially ventilated. Extracellular recordings of neuronal activity were made from the dorsal horn neurons in the lumbar spinal cord (L4–L5), using a recording glass microelectrode with a carbon filament (3–5 M Ω) while mechanical stimuli were applied onto the receptive fields. The single unit responses of the dorsal horn neurons, characterized as wide dynamic range (WDR) neurons by their graded responses to increased intensities of mechanical stimuli, were amplified, filtered, and displayed on an oscilloscope. The output signals were also fed into a data acquisition system (CED 1401 plus) via a window discriminator for the construction of real-time recordings of peristimulus time histograms, which were displayed as the number of spikes per second.

The mechanical stimuli were applied for 10 s and included: (1) brushing the skin with a camel hair brush in a stereotypic manner (brush); (2) sustained application of a large clamp that produced a sense of firm pressure when placed on human skin (pressure); and (3) sustained application of a small clamp that produced a distinctly painful sensation (pinch).

EVALUATION

Statistical analysis was performed using Mann-Whitney's unmatched pairs rank-sum test to evaluate the differences between the scores in two groups. All data are displayed as means \pm standard error.

MODIFICATIONS OF THE METHOD

Spinal cord injury often leads to central pain syndrome including hyperalgesia to mechanical stimulation. Several authors studied the influence of nerve growth factor in **models of neuropathic pain** (Ramer and Bisby 1999; Li et al. 2002, 2003; Cahill et al. 2003; Ruiz et al. 2004).

Nerve Ligation Injury

Nerve ligation injury was performed according to the method described previously (Kim and Chung 1992). This technique produces signs of tactile allodynia and thermal hyperalgesia. Rats were anesthetized with halothane and the L5 and L6 spinal nerves were exposed, carefully isolated, and tightly ligated with 4-0 silk suture distal to the dorsal root ganglion (DRG). After ensuring homeostatic stability, the wounds were sutured, and the animals were allowed to recover in individual cages. Sham-operated

rats were prepared in an identical fashion except that the L5 and L6 spinal nerves were not ligated.

Intrathecal Catheter Placement

Two routes of administration, a systemic i.p. and a spinal intrathecal (i.th.) route, were used to explore the activity of compounds. For the spinal route, test compounds were injected through indwelling i.th. catheters in the manner described by Yaksh and Rudy (1976). While under anesthesia, polyethylene tubing 10 tubing (8 cm) was inserted through an incision made in the atlanto-occipital membrane to the level of the lumbar enlargement of the rat and secured. Drug injections were made in a volume of 5 μ l followed by a 9- μ l saline flush.

Thermal Sensitization

Rats were lightly anesthetized with ether. The left hindpaw was placed in a water bath maintained at 50°C for 1 min. Inflammation suggested by rubor of the paw developed immediately. The rats were allowed to recover from anesthesia and tactile testing was begun 2 h after thermal sensitization. This procedure has produced signs of thermal hyperalgesia and tactile allodynia that persisted for over 12 h.

Evaluation of Tactile Allodynia

Mechanical allodynia was determined in the manner described previously (Chaplan et al. 1994). The paw withdrawal threshold was determined in response to probing with calibrated von Frey filaments. The rats were kept in suspended cages with wire mesh floors and the von Frey filaments were applied perpendicularly to the planar surface of the paw of the rat until it bent slightly, and was held for 3–6 s, or until the paw was withdrawn. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the non-parametric method (Dixon 1980).

Shelton et al. (2005) found that nerve growth factor mediates **hyperalgesia and cachexia in autoimmune arthritis**. Function-blocking antibodies to NGF completely reverse established pain in rats with fully developed arthritis despite continuing joint destruction and inflammation. Likewise, these antibodies reverse weight loss while not having any effect on levels of the pro-cachectic agent tumor necrosis factor (TNF).

Banik et al. (2005) reported that increased nerve growth factor after rat plantar incision contributes to **guarding behavior and heat hyperalgesia**. The therapeutic effect of a monoclonal antibody against en-

dogenous NGF was evaluated by intraperitoneal administration of a single preoperative dose of anti-NGF.

Adult male, 225–275 g, Sprague-Dawley rats were used in a plantar incision animal model. The animals were anesthetized with 1.5%–2% halothane and the surgical field was prepared in a sterile manner. A 1-cm longitudinal incision was made in the plantar aspect of the hind paw beginning 0.5 cm from the end of heel; skin, fascia and muscle were incised and the skin was closed with 5–0 nylon suture. Topical antibiotics were administered. On the second postoperative day, sutures were removed under brief anesthesia.

For measuring guarding behaviors, unrestrained rats were placed on a small plastic mesh floor (grid 8 \times 8 mm). Using an angled magnifying mirror, the incised and non-incised paws were viewed. Both paws of each animal were closely observed over a 1-min period repeated every 5 min for 1 h. Depending on the position in which each paw was found during the majority of the 1-min scoring period, a 0, 1 or 2 was given. A score of 0 was given for full weight bearing with the area of the wound blanched or distorted by the mesh; 1 for the wound area just touching the mesh without blanching or distortion; and 2 for the wound area completely off the mesh. The sum of the 12 scores (0–24) obtained during 1-h session for each paw was obtained.

For measuring heat sensitivity rats were placed individually on a glass floor covered with a clear plastic cage and allowed to acclimate. Withdrawal latencies to radiant heat were assessed by applying a focused radiant heat source underneath a glass floor on the middle of the incision. The latency time to evoke a withdrawal was determined with a cut-off value of 30 s. The intensity of the heat was adjusted to produce a withdrawal latency in normal rats of 25–30 s. Each rat was tested at least three times, at an interval of 10 min. The average of at least three trials was used to obtain paw withdrawal latency.

For measuring mechanosensitivity, rats were placed individually on a plastic mesh floor covered with a clear plastic cage and allowed to acclimate. The withdrawal response to punctate mechanical stimulation was determined using calibrated von Frey hairs applied underneath the cage to an area adjacent to the incision. Each filament was applied once starting with 10 mN and continuing until a withdrawal response occurred or 250 mN was reached. If a rat did not respond to the 250-mN filaments (522 mN), the next filament was recorded. This was repeated a total of three times with at least a 5- to 10-min test-free period between withdrawal responses. The lowest force from the three

tests producing a response was considered the withdrawal threshold.

Zahn et al. (2004) described the effect of blockade of nerve growth factor and tumor necrosis factor on pain behaviors after plantar incision.

Obata et al. (2002) described the expression of neurotrophic factors in the dorsal root ganglion in a **rat model of lumbar disc herniation**. The left L4/5 nerve roots were exposed after hemilaminectomies and autologous intervertebral discs, which were obtained from coccygeal intervertebral discs, were implanted on each of the exposed nerve roots without mechanical compression.

Lamb et al. (2003) studied nerve growth factor (NGF) and **gastric hyperalgesia** in the rat. Male Sprague Dawley rats (300–400 g) were anesthetized and the stomach exposed and placed in a circular clamp. Acetic acid (60%) or saline was injected into this area and aspirated 45 s later, resulting in kissing ulcers. A balloon was surgically placed into the stomach and electromyographic responses to gastric distension recorded from the acromiotrapius muscle. Animals received a daily injection of neutralizing NGF antibody or control serum for 5 days; NGF in the stomach was measured with an ELISA. The severity of gastric injury was assessed microscopically and by determination of myeloperoxidase activity.

Winston et al. (2003) investigated molecular and behavioral changes in nociception in a novel **rat model of chronic pancreatitis** induced by pancreatic infusion of trinitrobenzene sulfonic acid as a model of painful pancreatitis. Nociception was assessed by measuring mechanical sensitivity of the abdomen and by recording the number of nocifensive behaviors in response to electrical stimulation of the pancreas. Expression of neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) in the thoracic dorsal root ganglia receiving input from the pancreas and nerve growth factor in the pancreas were measured.

Guerios et al. (2006) reported that nerve growth factor (NGF) mediates peripheral mechanical hypersensitivity that accompanies **experimental cystitis in mice**. Cystitis was induced by intraperitoneal injection of cyclophosphamide (CYP) in female mice. Sensitivity of hind paws to mechanical stimuli was determined prior to and 4, 9, and 24 h after CYP, and the sensitivity of the tail to thermal stimuli was determined prior to, and 4 and 24 h after CYP treatment. To investigate the role of NGF in these processes, other groups of mice received NGF antiserum or normal serum intravenously 30 min after CYP administration. CYP induced blad-

der inflammation that was not ablated by treatment with NGF antiserum. Sensitivity to mechanical stimuli was increased 4 and 9 h after CYP administration. This was reversed by NGF antiserum but not by normal serum.

Cyclophosphamide-induced cystitis was also used as a model for visceral pain by Lanteri-Minet et al. (1995), Boucher et al. (2000), and Bon et al. (2003).

Jaggat et al. (1999) studied hyperalgesia to thermal stimulation of the hind limb of rats after inflammation of the urinary bladder by instillation of 0.5 ml of 50% turpentine in olive oil.

Dmitrieva and McMahon (1996) and Dmitrieva et al. (1997) reported sensitization of visceral afferents by NGF in the adult rat.

Delafoy et al. (2003) studied the role of NGF in **trinitrobenzene sulfonic acid-induced colonic hypersensitivity**. The function of NGF as a mediator of persistent pain states was tested in a model of colonic hypersensitivity measured by isobaric distension in conscious rats. The effects of exogenous NGF on colonic pain threshold, the involvement of NGF in trinitrobenzene sulfonic acid-induced colonic hypersensitivity, and the involvement of sensory nerves in the effects of NGF and trinitrobenzene sulfonic acid using rats treated neonatally with capsaicin were studied.

Sevcik et al. (2005) found that anti-NGF therapy profoundly reduced **bone cancer pain** and the accompanying increase of markers of peripheral and central sensitization. Osteolytic murine sarcoma cells were injected into the intramedullary space of the mouse femur. Administrations of a NGF-sequestering antibody produced a profound reduction in cancer pain-related behavior that was greater than that achieved with administration of morphine.

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H.3 Anti-Inflammatory Activity

H.3.0.1 General Considerations

Inflammation was characterized two thousand years ago by Celsus by the four Latin words: Rubor, calor, tumor and dolor. Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation and chronic repair processes. In some cases, the screening is directed to test compounds for local application. Predominantly, however, these studies are aimed to find new drugs against polyarthritis and other rheumatic diseases. Since the etiology of polyarthritis is considered to be largely immunologically, special tests have been developed to investigate various immunological and allergic factors (see Chapter I).

H.3.1 In Vitro Methods for Anti-Inflammatory Activity

H.3.1.1 General Considerations

An array of physiological substances, sometimes called autacoids, are involved in the process of inflammation and repair. These include histamine, serotonin,

bradykinin, substance P, and the group of eicosanoids (prostaglandins, thromboxanes and leucotrienes), the platelet-activating factor (PAF) as well as cytokines and lymphokines. Their discovery makes the use of *in vitro* studies possible. The influence of non-steroidal anti-inflammatory agents on the eicosanoid pathway gave rise to numerous studies.

H.3.1.2

³H-Bradykinin Receptor Binding

PURPOSE AND RATIONALE

Tissue injury or trauma initiates a cascade of reactions which results in the proteolytic generation of bradykinin and kallidin from high-molecular-weight precursors, kininogens, found in blood and tissue. The rapid enzymatic cleavage of kininogens is accomplished by the kallikreins, a group of proteolytic enzymes which are present in most tissues and body fluids. Bradykinin produces pain by stimulating A and C fibers in the peripheral nerves, participates in the inflammatory reaction and lowers blood pressure by vasodilatation. Since its breakdown occurs via the same enzyme responsible for converting angiotensin I into angiotensin II some of the effects of converting enzyme inhibitors may be due to presence of bradykinin. The ³H-bradykinin receptor binding is used to detect compounds that inhibit binding of ³H-bradykinin in membrane preparations obtained from guinea-pig ileum. Two types of bradykinin receptors (B₁ and B₂ receptors) are known (Feres et al. 1992; Bascands et al. 1993; Tropea et al. 1994; Marceau et al. 1998; Calixto et al. 2004; Leeb-Lundberg et al. 2005). The existence of a pulmonary BK₃ receptor has been proposed by Farmer et al. (1989) and Meini et al. (2004). Evidence was obtained for the existence of three subtypes of B₂ receptors, B_{2a}, B_{2b}, and B_{2c} (Seguin et al. 1992; Seguin and Widdowson 1993).

PROCEDURE

Ileum from guinea pigs is cleaned from its content and cut into pieces of 2 cm length. They are homogenized for 30 s in ice-cold TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, in a Potter homogenizer. The homogenates are filtered through 3 layers of gauze and centrifuged twice at 50,000 *g* for 10 min with an intermediate rehomogenization in buffer.

For routine studies the final pellets are resuspended in 40 vol of incubation buffer (25 mM TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, 0.1% bovine serum albumin, 140 μg/ml bacitracin, 1 mM dithiothreitol, 0.1 μM captopril). In the competition

experiment, 50 μl ³H-bradykinin (one constant concentration of 0.5–2 × 10⁻⁹ M), 50 μl test compound (6 concentrations, 10⁻⁵–10⁻¹⁰ M) and 150 μl membrane suspension from guinea pig ileum (approx. 6.6 mg wet weight/ml) per sample are incubated in a shaker bath at 25°C for 90 min.

Saturation experiments are performed with 12 concentrations of ³H-bradykinin (14.2–0.007 × 10⁻⁹ M). Total binding is determined in the presence of incubation buffer, non-specific binding is determined in the presence of non-labeled bradykinin (10⁻⁶ M).

The reaction is stopped by rapid vacuum filtration through glass fibre filters. Thereby the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding of ³H-bradykinin
- non-specific binding in the presence of 10 μM bradykinin
- specific binding = total binding – non-specific binding
- % inhibition: 100 – specific binding as percentage of control value

Compounds are first tested at a single high concentration (10,000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is constructed using 7 different concentrations of test compound. Binding potency of compounds is expressed either as a relative binding affinity (*RBA*) with respect to the standard compound (bradykinin) which is tested in parallel or as an *IC*₅₀.

$$RBA = \frac{IC_{50} \text{ standard compound}}{IC_{50} \text{ compound}} \times 100\%$$

The dissociation constant (*K_i*) and the *IC*₅₀ value of the test drug are determined from the competition experiment of ³H-bradykinin versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

Tests for Bradykinin Receptor Types and Subtypes

Prado et al. (2002) described mechanisms regulating the expression, self-maintenance, and signaling function of the bradykinin B₂ and B₁ receptors.

Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor was reported by Hess et al. (1992).

Menke et al. (1994) reported the expression cloning of a human bradykinin B₁ receptor.

Bradykinin B₁ receptors have been studied in the isolated rabbit aorta (Bouthillier et al. 1987), in the isolated rabbit carotid artery (Pruneau and Bélichard 1993) and in the rabbit urinary bladder (Butt et al. 1955).

A potent bradykinin B₁ receptor antagonist has been described by Wirth et al. (1991).

Heterogeneity of B₁ receptors has been suggested by Wirth et al. (1992).

Heitsch (2002) reviewed non-peptide antagonists and agonists of the bradykinin B₂ receptor.

Bradykinin receptor ligands were described by Marceau and Regoli (2004) and Fortin and Marceau (2006).

Drummond and Cocks (1995) used rings of bovine left anterior descending coronary artery to study endothelium-dependent relaxations mediated by inducible B₁ and constitutive B₂ kinin receptors.

The production of cyclic GMP via activation of B₁ and B₂ kinin receptors in cultured bovine aortic endothelial cells was described by Wiemer and Wirth (1992).

Pharmacological characterization of bradykinin receptors in canine cultured tracheal smooth muscle cells has been reported by Yang et al. (1995).

Bradykinin B₂ receptors and their antagonists have been studied in human fibroblasts by Alla et al. (1993), with the high affinity radioligand [¹²⁵I]PIP HOE 140 by Brenner et al. (1993), in guinea pig gall bladder by Falcone et al. (1993), in the smooth muscle of guinea-pig taenia caeci by Field et al. (1994), in guinea pig ileum membranes by Graneß and Liebmann (1994), Liebmann et al. (1994a), in isolated blood vessels from different species by Félétou et al. (1994), in endothelial cells by Wirth et al. (1994).

Hallé et al. (2000) described *in vitro* and *in vivo* effects of kinin B₁ and B₂ receptor agonists and antagonists in inbred control and cardiomyopathic hamsters.

The role of B₁ and B₂ receptors and of nitric oxide in bradykinin-induced relaxation and contraction of isolated rat duodenum was studied by Rhaleb and Carretero (1994).

Campos et al. (1996) investigated the effect of pretreatment with bacterial endotoxin on the bradykinin B₁ and B₂ receptor-induced edema in the rat paw and the interaction of B₁-mediated responses with other inflammatory mediators.

Characterization of kinin receptors by bioassays was described by Gobeil and Regoli (1994). Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B₂ receptor gene was performed by Eggerickx et al. (1992).

Simpson et al. (2000) characterized bradykinin analogues on recombinant human bradykinin B₁ and B₂ receptors using a high throughput functional assay which measures intracellular Ca²⁺ responses.

Bradykinin B₂ receptor subtypes were discussed by Liebmann et al. (1994b) and Regoli et al. (1994).

Evidence for a pulmonary B₃ bradykinin receptor has been given by Farmer et al., (1989) and Meini et al. (2004).

Bradykinin B₃ receptors have been described by Field et al. (1992) in the smooth muscle of the guinea-pig taenia caeci and trachea.

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H.3.1.3

Substance P and the Tachykinin Family

H.3.1.3.1

General Considerations

Substance P belongs to the tachykinin family of peptides that share a common carboxy-terminal sequence (Phe-X-Gly-Leu-Met-NH₂). It was first described by von Euler and Gaddum (1931) as a brain and gut extract that stimulates smooth muscle contraction. Bioassay extracts from spinal dorsal roots implicated substance P as a pain neurotransmitter (Lembeck 1953; Lembeck and Holzer 1979). After determination of the amino acid sequence (Chang et al. 1971) the distribution of substance P in the CNS could be studied (Hökfelt et al. 1975). Neurokinins belong like substance P to a group of neuropeptides named tachykinins. Following the discovery of neurokinin A and neurokinin B, three distinct G protein-coupled receptors, NK₁, NK₂ and NK₃, were described (Maggi et al. 1993; Mussap et al. 1993; Patacchini and Maggi 1995). Neurokinin A and substance P are preferred agonists of the tachykinin NK₁ and NK₂ receptors, whereas neurokinin B preferentially interacts with the tachykinin NK₃ receptor. The receptor sensitivity of these peptides is relatively poor, and it is possible that their actions could be mediated by interactions with their less preferred receptors.

Nomenclature of tachykinins and tachykinin receptors has been discussed repeatedly (Henry 1987; Maggi 2000).

Tachykinin NK₁ antagonists are potent antiemetics, however other possible therapeutic uses, including rheumatoid arthritis, asthma, migraine, pain and psychiatric disorders, were suggested (Longmore et al. 1995). The P-preferring NK₁ receptor has attracted most interest as a CNS target because it is the predominant tachykinin receptor expressed in the human brain, while NK₂ and NK₃ receptor expression is in extremely low abundance or absent. Several NK₁ receptor agonists antagonists were synthesized and evaluated (Snider et al. 1991; Emonds-Alt et al. 1993; Cascieri et al. 1992; Sakurada et al. 1993; Bristow and Young 1994; Jung et al. 1994; Rupniak and Williams 1994; Smith et al. 1994; Vassout et al. 1994; Patacchini and Maggi 1995; Bonnet et al. 1996; Chapman et al. 1996; Herbert and Bernat 1996; Palframan et al. 1996; Ren et al. 1996). Moreover, agonists and antagonists at the NK₂ receptor (Hagan et al. 1991, 1993; Beresford et al. 1995; Robineau et al. 1995; Kudlacz et al. 1997; Lecci et al. 1997) and at the NK₃ receptor (Guard et al. 1990; Edmonds-Alt et al. 1995; Patacchini et al. 1995; Nguyen-Le et al. 1996; Beaujouan et al. 1997; Sarau et al. 1997) were reported (Longmore et al. 1995; Rupniak 1999).

Understanding the role of substance P in the brain has been complicated by marked species differences in the distribution of the tachykinin receptor types. There appears to be a relative increase in NK₁ receptor density during evolution, such that NK₃ receptors are abundant in lower vertebrates and mammals but, like NK₂ receptors, are apparently absent in human brain. Preclinical studies with substance P receptor antagonists have been hindered not only by phylogenetic differences in tachykinin receptor expression, but also by pharmacological heterogeneity of the NK₁ receptor and the NK₂ receptor. An other confounding feature of neurokinin receptor antagonist is the blockade of Na⁺ and Ca²⁺ channels at high doses which produces effects in various assays that are independent of receptor antagonism (Patacchini and Maggi 1995; Rupniak 1999). Most developments were guided by the effects of substance P as a pain neurotransmitter. Surprisingly, most clinical studies of analgesic activity of NK₁ receptor antagonists were negative (Rupniak and Kramer 1999). However, clinical findings indicated that substance P receptor antagonists are able to alleviate depression and anxiety in patients suffering from major depressive disorder (Kramer et al. 1998).

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H.3.1.3.2

³H-Substance P Receptor Binding

PURPOSE AND RATIONALE

Substance P is an undekapeptide which is widely distributed in the central and peripheral nervous systems and functions as a neurotransmitter/neuromodulator in a variety of physiological processes. Substance P is released from neurons in the midbrain in response to stress where it facilitates dopaminergic neurotransmission and from sensory neurons in the spinal cord in the response to noxious stimuli, where it excites dorsal neurons. In the periphery, release of substance P from sensory neurons causes vasodilatation and plasma extravasation, suggesting a role in neurogenic inflammation. Selective antagonists to substance P found in receptor binding studies may elucidate the physiological role of substance P and may be candidates for anti-inflammatory and analgesic drugs.

PROCEDURE

Fresh porcine brains are obtained from the slaughterhouse. Striata are dissected and homogenized (Ultraturrax) in 50 mM ice-cold Tris-HCl buffer, pH 7.4) containing 150 mM NaCl, 150 mM KCl, 12 mM EDTA, 200 μM phenylmethylsulfonyl fluoride, 40 μg/ml bacitracin, 4 μg/ml leupeptin, and 2 μg/ml chymostatin. These homogenates are then incubated for 30 min at 4°C before being centrifuged at 30,000 g for 20 min at 4°C and washed twice with 50 mM Tris-HCl (pH 7.4) buffer. Pellets are resuspended in 0.32 M sucrose containing 200 μM phenylmethyl sulfonylflu-

oride and 40 μg/ml bacitracin before storage at –80°C until use.

Sixty-minute incubations are carried out at room temperature in 50 mM Tris-HCl buffer, pH 7.4, containing various concentrations of [³H]substance P ([³H]SP) (0.05–20 nM), 5 mM MgSO₄, 40 mg/ml bacitracin, 4 mg/ml leupeptin, and 2 mg/ml chymostatin in the presence of 0.8–1 mg of membrane protein in a final volume of 1 ml. Total binding and nonspecific binding are determined in triplicate in the absence or presence of 1 mM unlabeled substance P. Incubations are terminated by adding 4 ml of ice-cold Tris-HCl buffer (pH 7.4) and membranes are filtered on Whatman glass fiber filters that are presoaked in 0.5% polyethylenimine for a minimum of 3 h to reduce absorption. Filters are then washed three times (5 ml each) using ice-cold Tris-HCl buffer (pH 7.4). Bound radioactivities are determined using a liquid scintillation counter.

EVALUATION

Saturation and competition data are analyzed using a computer program as described by McPherson (1985).

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H.3.1.3.3

Neurokinin Receptor Binding

PURPOSE AND RATIONALE

The actions of tachykinins are mediated through three subtypes of neurokinin receptors belonging to the G protein-linked receptor family, namely, NK₁, NK₂ and NK₃. Substance P displays highest affinity to NK₁

receptors, whereas neurokinin A and neurokinin B preferably bind to NK₂ and NK₃ receptors, respectively. NK₁ receptors are expressed in a wide variety of peripheral tissues and in the CNS. NK₂ receptors are expressed primarily in the periphery, while NK₃ receptors are primarily expressed in the CNS.

PROCEDURE

Tachykinin NK₁ receptor binding assay is performed in intact Chinese hamster ovary (CHO) cells expressing the human tachykinin NK₁ receptor (Cascieri et al. 1992). The receptor is expressed at a level of 3×10^5 receptors per cell. Cells are grown in a monolayer culture, detached from the plate with enzyme-free cell dissociation solution (Specialty Media), and washed prior to use in the assay. ¹²⁵I[Tyr⁸]substance P (0.1 nM, 2000 Ci/mmol; New England Nuclear) is incubated in the presence or absence of test compounds (dissolved on 5 μ l DMSO) with 5×10^4 CHO cells. Ligand binding is performed in 0.25 ml of 50 mM Tris-HCl, pH 7.5, containing 5 mM MnCl₂, 150 mM NaCl, 0.02% bovine serum albumin, 40 μ g/ml bacitracin, 0.01 mM phosphoramidon and 4 μ g/ml leupeptin. The incubation proceeds at room temperature until equilibrium is achieved (>40 min) and the receptor ligand complex is harvested by filtration over GF/C filters presoaked in 0.1% polyethylenimine using a Tomtek 96-well harvester. Nonspecific binding is determined using excess substance P (1 μ M) representing <10% of total binding.

For **NK₂receptor binding assays** membranes of CHO cells transfected with human ileum NK₂ receptor are used (Hagan et al. 1993; Beresford et al. 1995). The membrane suspensions (5 μ g protein) in assay buffer (Tris base (50 mM), MnCl₂ (3 mM), bovine serum albumin (0.05%), chymostatin (2 μ g/ml) and leupeptin 4 μ g/ml, pH 7.4) are incubated for 90 min at room temperature with wash buffer (Tris base (50 mM), MnCl₂ (3 mM), lauryl sulphate (0.01%), pH 7.4) or test compound, and [³H]-GR100679 (0.5 nM final concentration). Non-specific binding is defined by use of GR159897 (1 μ M).

For **NK₃ receptor binding assays** guinea pig cortical membranes (Guard et al. 1990) are incubated at room temperature for 60 min with HEPES wash buffer or test compound and [³H]-senktide (final concentration 0.8–1.0 nM). Non-specific binding is defined by addition of eledoisin (10 μ M).

EVALUATION

Inhibition curves are analyzed and pIC_{50} values calculated by use of a curve fitting program. pIC_{50} values

are converted to inhibition constants (pK_i values) using the Cheng Prussoff equation

$$K_i = IC_{50}/(1 + L/K_D)$$

where L is the ligand concentration and K_D is the dissociation constant. The K_D and B_{max} (maximum number of binding site per mg of tissue) are determined from saturation curves and analyzed by a curve fitting program. Values are expressed as means \pm SEM.

MODIFICATIONS OF THE METHOD

Watson et al. (1955) performed substance P binding assays in **ferret brain membranes** and assessed neurokinin NK₁ receptor binding using human lymphoblasts (IM-9 cells).

Rupniak et al. (1997) studied displacement of [¹²⁵I]-[Tyr⁸]substance P binding to cloned human tachykinin NK₁ receptors and to ferret brain membranes *in vitro*.

Beattie et al. (1995) used U373 MG cell membranes and cerebral cortical membranes from rat, ferret and gerbil and [³H]substance P for NK₁ receptor binding assays.

Bonnet et al. (1996), McLean et al. (1996) used the IM9 lymphoblastoma cell line expressing the human NK₁ receptor.

Emonds-Alt et al. (1995) studied binding of [¹²⁵I] Bolton-Hunter labelled substance P to **NK₁receptors** of rat brain cortex, human lymphoblast cells (IM9), and human astrocytoma cells (U373MG, STTG1), binding of [¹²⁵I]iodohistidyl-NKA (or [¹²⁵I]neuropeptide γ) to **NK₂receptors** of rat or hamster urinary bladder or guinea pig ileum, binding of [¹²⁵I]iodohistidyl-[Me-Phe⁷]NKB (or [¹²⁵I]Eledoisin) to tachykinin **NK₃ receptors** of rat, guinea pig and gerbil brain cortex., and of [¹²⁵I]iodohistidyl-[Me-Phe⁷]NKB to the human NK₃ receptor, cloned and expressed in CHO cells (Buell et al. 1992).

Cascieri et al. (1992) described the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor.

A radioligand of the tachykinin NK₂ receptor was described by Renzetti et al. (1998).

Jordan et al. (1998) evaluated the Cytosensor microphysiometer, a system that measures the extracellular acidification rate as an index of the integrated functional response to receptor activation, as a method to study NK₃ receptor pharmacology and used this system to assess the functional activity of novel compounds at this receptor.

Appell et al. (1998) reported biological characterization of neurokinin antagonists discovered through

screening of a combinatorial library. Using stably transfected CHO-K1 cell lines expressing human NK-1, NK-2 and NK-3 receptor subtypes and europium time-resolved fluorescence, primary receptor binding assays were designed to define active compounds. In addition, a secondary, functional assay measuring intracellular calcium flux with the calcium-sensitive fluorophore, fluo-3, in CHO cells transfected with the human NK-1 or NK-2 receptor was used to determine agonist or antagonist activities.

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H.3.1.3.4 Characterization of Neurokinin Agonists and Antagonists by Biological Assays

PURPOSE AND RATIONALE

Several biological assays have been used to characterize neurokinin agonists and antagonists on their receptors (Review by Regoli et al. 1994).

The following functional assays are recommended for evaluation of antagonists:

For NK₁ Receptors

- Inhibition of [Sar⁹,Met(O₂)¹¹]substance P-induced endothelium-dependent **relaxation of rabbit pulmonary artery**, previously contracted with 0.1 μM noradrenaline (D'Orléans-Juste et al. 1986; Rubino et al. 1992; Emonds-Alt et al. 1993),
- inhibition of [Sar⁹,Met(O₂)¹¹]substance P or [Sar⁹]substance P sulfone-induced contractions of **guinea pig ileum** in the presence of 3 μM atropine and 3 μM mepyramine and indomethacin (Dion et al. 1987; Emonds-Alt et al. 1993; Patacchini et al. 1995; Hosoki et al. 1998; Walpole et al. 1998),
- **rabbit vena cava** stimulated by substance P or [Sar⁹,Met(O₂)¹¹]substance P (Nantel et al. 1991; Regoli et al. 1994; Gitter et al. 1995; Robineau et al. 1995; Bonnet et al. 1996; Nguyen-Le et al. 1996),
- inhibition of substance P-induced **relaxation of the isolated dog carotid artery** previously contracted with norepinephrine (Snider et al. 1991),
- **rat urinary bladder**, stimulated by the selective agonist [Sar⁹,Met(O₂)¹¹]substance P and treated with SR 48986 (1.7×10^{-7} mol/l) to eliminate NK-2 functional sites (Rouissi et al. 1993; Nguyen-Le et al. 1996),
- Ca²⁺ mobilization in **rat vas deferens** (Nagata et al. 1991),
- inhibition of **substance P-induced plasma extravasation in the bladder and bronchi** of the guinea pig (Bonnet et al. 1996),
- inhibition of **substance P-induced vasodilation in the nasal mucosa of pigs** using an acoustic rhinometer (Rinder and Lundberg 1996),
- inhibition of [Sar⁹,Met(O₂)¹¹]substance P-induced **plasma extravasation in guinea-pig bronchi** (Cirillo et al. 1998),
- inhibition of **methacholine-induced contractions of isolated rat tracheal strips** (Tian et al. 1997),
- inhibition of **cyclophosphamide- and radiation-induced damage in the rat and ferret organs** (Alfieri and Gardner 1997, 1998),
- inhibition of **edema formation induced by substance P and antigen in rat skin** (Herbert and Bernat 1996),
- inhibition of **reciprocal hindlimb scratching after intracerebroventricular injection of substance P, [Sar⁹,Met(O₂)¹¹]substance P or septide in mice** (Jung et al. 1994) or gerbils (Smith et al. 1994),
- inhibition of **turning behavior after intracerebroventricular injection of substance P, [Sar⁹,Met(O₂)¹¹] substance P or septide in mice** (Jung et al. 1994),

- inhibition of **hind paw tapping and chromodacry-orrhoea after intracerebroventricular injection of tachykinin agonists in gerbils** (Graham et al. 1993; Bristow and Young 1994; Rupniak and Williams 1994; Rupniak et al. 1995, 1997; Vassout et al. 1994),
- inhibition of **cis-platin-induced emesis in ferrets** (Rupniak et al. 1997; Singh et al. 1997; Minami et al. 1998).

For NK₂ Receptors

- Inhibition of neurokinin A-induced contraction of **isolated rabbit aorta** (Snider et al. 1991),
- inhibition of neurokinin A-induced contraction of **isolated endothelium-deprived rabbit pulmonary artery or hamster trachea** (D'Orléans-Juste et al. 1986; Emonds-Alt et al. 1993; Patacchini et al. 1995),
- the **hamster urinary bladder** (Dion et al. 1987; Maggi et al. 1990; Regoli et al. 1994; Emonds-Alt et al. 1997; Tramontana et al. 1998),
- inhibition of **motor responses induced by intravesical administration of capsaicin** in rats *in vivo* (Lecci et al. 1997),
- **rat esophageal tunica muscularis** (Crocini et al. 1995),
- inhibition of **turning behavior induced by intratriatal injection of Nle¹⁰-neurokinin A in mice** (Emonds-Alt et al. 1997).

For NK₃ Receptors

- Inhibition of **senktide- or neurokinin B-induced contractions of the rat portal vein** (Mastrangelo et al. 1987; Snider et al. 1991; Emonds-Alt et al. 1993; Patacchini et al. 1995),
- antagonism against **senktide-induced contractions in the isolated rabbit iris sphincter muscle** (Medhurst et al. 1997; Sarau et al. 1997),
- inhibition of **colonic propulsion in rats** (Broccardo et al. 1999)
- inhibition of **turning behavior induced by intratriatal injection of senktide in gerbils** (Emonds-Alt et al. 1994),
- inhibition of **citric acid-induced cough in guinea pigs** (Daoui et al. 1998).
- The failure of NK₁ receptor antagonists in most clinical tests for analgesia in spite of clear preclinical data is a matter of discussion (Hill R 2000a, b; Urban and Fox 2000).

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H.3.1.4

Assay of Polymorphonuclear Leukocyte Chemotaxis In Vitro

PURPOSE AND RATIONALE

Leukocyte accumulation is an important aspect of host defense mechanisms. Chemotactic factors attract leukocytes to an infected or inflamed site. The method of Boyden (1962) has been widely employed to measure the chemotactic effects on polymorphonuclear leukocytes. Watanabe et al. (1989) described a rapid assay of polymorphonuclear leukocyte chemotaxis *in vitro*.

PROCEDURE

Two 96-well tissue-culture plates are utilized as one set of multiple Boyden chambers. One plate as multiple lower compartments and the other plate upside down as multiple upper compartments can be tightly sandwiched with the aid of 12 sets of bolts. The holes, into which bolts are set and polymorphonuclear leukocytes (PMN) suspensions are introduced, are opened by a heated stick. Eight sets of 6 holes are made on the bottom of the upper plate to serve as multiple upper compartments for introducing PMN suspensions. Eight sets of polycarbonate filter (approximately 3.2 × 2.2 cm), cut from a round filter (Nuclepore Co.) with pores of 2 μm in diameter, are sandwiched between the upper and lower plates. One sheet of the filter can separate six sets of the upper compartments from the lower ones. Silicon grease is spread on all the plate surfaces that attach to the filters. The lower compartments are filled with a chemoattractant (400 μl/well) diluted in RPMI-1640 medium. The eight sheets of filter paper are carefully placed on each set of the lower compartments to avoid air bubbles. The upper plate is positioned over the lower plate and fastened with bolts. The upper compartments are filled with 0.3 ml of PMN suspension (at 10⁷ cells/ml). The assembly is incubated at 37°C for 60 min in a humidified atmosphere containing 5% CO₂. Then the fluid in the upper compartment is decanted and the upper compartments are completely washed with a jet of water. The lower plate is centrifuged at 2400 rpm for 5 min at room temperature and the supernatant in the well is decanted. The pellet of PMNs is dispersed in 200 μl of phosphate buffered saline containing 0.1% EDTA. Absorbance at 660 nm of each well containing PMN suspension is determined with a 96-well microplate reader. The number of PMNs in the lower compartments is further determined by a Coulter counter.

EVALUATION

The migration rate is calculated as percentage from the number of PMNs in the lower compartment/number of PMNs applied in the upper compartment. The migration rate is dependent on the concentration of the chemoattractant (e. g., zymosan-activated serum). Moreover, a dose dependent decrease of migration rate is achieved by chemotaxis inhibitors.

MODIFICATIONS OF THE METHOD

Nelson et al. (1975) described chemotaxis under agarose as a simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes.

Migration of PMNs in agarose gel was measured after fixation with glutaraldehyde and staining with Giemsa by Shalaby et al. (1987).

Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood were described by Ferrante and Thong (1980).

PMN chemotaxis was measured in multiwell microchemotaxis chambers separated by 5- μ m pore size polyvinylpyrrolidone-free polycarbonate membranes by Harvath et al. (1980), Figari et al. (1987).

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H.3.1.5

Polymorphonuclear Leukocytes Aggregation Induced by FMLP

PURPOSE AND RATIONALE

Aggregation of polymorphonuclear leukocytes (PMNs) can be induced by FMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine). The aggregation can be inhibited by xanthine derivatives.

PROCEDURE

PMNs cell suspensions are prepared from peritoneal exudates obtained 17 h after intraperitoneal injection of 10 ml 6% sodium caseinate into Sprague Dawley rats. The cells are washed twice in Geys-balanced-salt-solution (Gibco GBSS) and resuspended to a final concentration of 15×10^6 cells/ml. The test compounds and the standard (pentoxifylline) are dissolved in GBSS. FMLP (formyl-L-methionyl-L-leucyl-L-phenylalanine) is dissolved in DMSO. The further dilutions are made up to a final concentration of 10^{-7} mol FMLP in GBSS. Before addition of FMLP, the cell suspensions are pre-incubated for 10 min with the drugs. PMNs aggregation is carried out in a Born aggregometer.

EVALUATION

The results are expressed as change in transmittance, measured in mm on the recorder. The mean peak of the untreated cells is set 100%.

MODIFICATIONS OF THE METHOD

Moqbel et al. (1986) measured the activation of human leukocytes after FMLP in a rosette assay by the change in the expression of complement (C3b) and IgG (Fc)

receptors and in a cytotoxic assay by the *in vitro* capacity to adhere to and kill the complement-coated larvae (schistosomula) of *Schistosoma mansoni*.

Bradford and Rubin (1986) determined the effect of various drugs on IP₃ accumulation evoked by FMLP in neutrophils from New Zealand white rabbits.

Bourgoin et al. (1991) studied the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) on phosphatidylcholine breakdown by phospholipase D in human neutrophils.

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H.3.1.6

Constitutive and Inducible Cellular Arachidonic Acid Metabolism In Vitro

PURPOSE AND RATIONALE

The various metabolites of arachidonic acid are involved in many inflammatory processes. Arachidonic acid is released from the cellular phospholipid fraction by the action of phospholipase A₂, and subsequently metabolized via two major routes: the cyclooxygenase pathway yielding the primary prostaglandins and thromboxane, and the 5-lipoxygenase pathway yielding the leukotrienes. Thromboxanes, prostaglandins, and leukotrienes play a pathophysiological role in many diseases. Receptor assays for these autocooids were developed (Haluska et al. 1989). Murata et al. (1997) produced mice lacking the prostaglandin receptor which showed increased susceptibility to thrombosis and altered pain reception and inflammatory response.

The therapeutic mode of action of the classical non-steroidal anti-inflammatory drugs (NSAID), such as aspirin or indomethacin, is primarily explained by their inhibitory effect on cyclooxygenase, the key enzyme of the prostaglandin pathway. Inhibitors of the 5-lipoxygenase pathway have attracted considerable

attention as potential anti-inflammatories with high potency. Appropriate assay systems for the determination of the different eicosanoids allow to study the influence of drugs towards the specific pathways of the arachidonic acid cascade in various cellular systems (Samuelsson 1986; Vane and Botting 1987).

According to recent discoveries there are two forms of cyclooxygenase (Xie et al. 1992; Lee et al. 1992; Gierse et al. 1996). Cyclooxygenase-1 (COX-1) is found as a constitutive enzyme in most tissues including blood platelets. Prostaglandins generated by constitutive pathways may exert cytoprotective effects, and are involved in maintaining vital functions in vascular hemostasis, gastric mucosa and kidney.

Chandrasekharan et al. (2002) described the cloning, structure and expression COX-3, a cyclooxygenase-1 variant, which is selectively inhibited by analgesic/antipyretic drugs, such as acetaminophen, phenacetin, antipyrine, and dipyron.

The inhibition of these prostaglandins by the classical cyclooxygenase inhibitors is now generally accepted as an explanation of their adverse side effects.

COX-2 which shares about 62% amino acid homology with COX-1, is only expressed after cell activation, especially by mitogenic or inflammatory stimuli (Herrmann et al. 1990; Funk et al. 1991; Crofford 1997). Thus, specific suppression of the COX-2 pathway may represent a superior target for the evaluation of new antiinflammatory drugs. Drugs which have a high potency on COX-2 and a favorable COX-2/COX-1 ratio have potent anti-inflammatory activity with fewer side effects (Riendeau et al. 1997; Vane 1998; Hawkey 1999; Song et al. 1999; Chan et al. 1999). Shigeta et al. (1998) described the role of cyclooxygenase-2 (COX-2) in the healing of gastric ulcers in rats. Hull et al. (2005) investigated the expression of cyclooxygenase-1 and -2 by human gastric endothelial cells.

The cardiovascular value of selective COX-2 inhibitors has been questioned because they selectively reduce prostacyclin production, thus disrupting the hormonal balance and promoting a prothrombotic state (Hankey and Eikelboom 2003).

These theoretical concerns were supported by the results of clinical trials demonstrating an increased risk of myocardial infarction with COX-2 inhibitors compared with conventional non-steroidal anti-inflammatory drugs (NSAIDs) (Bombadier et al. 2000). The debate on benefit-risk assessment of COX-2 inhibitors is ongoing (Bing 2003; Schmidt et al. 2004).

H.3.1.6.1**Formation of Leukotriene B₄
in Human White Blood Cells In Vitro****PROCEDURE**

Human white blood cells are prepared according to the standard procedure published by Salari et al. (1984): 40 ml freshly drawn citrated blood are admixed with 8 ml of PM16-buffer, containing 6% (v/v) dextran (MW = 480,000), and incubated at room temperature for one hour. The supernatant containing the white blood cell fraction is removed, diluted 1:1 (v/v) with PM16 and centrifuged for 15 min at 300 g. The precipitate is resuspended in PM16 and adjusted to 10¹⁰ cells/l (Counter HT, Coulter Electronics, Krefeld, FRG).

The metabolism of endogenously bound arachidonic acid to LTB₄ is measured in a total volume of 0.3 ml of the cell suspension at 37°C. The reaction tube contains 2 mmol/l CaCl₂, 0.5 mmol/l MgCl₂ and the investigational drug. After 15 min pre-incubation the reaction is started by addition of 12.5 µg of the Ca-ionophore A 23187 and 2 µg glutathione. After 5 min the reaction is stopped with 30 µl 0.1 M HCl at 0°C. After centrifugation for 2 min at 0°C, aliquots of the supernatant are subjected to HPLC, similarly as described by Veenstra et al. (1988), using a C-18 Nucleosil column (5 µm, 100 × 3 mm, Chrompack GmbH, Frankfurt, FRG) and, at a flow rate of 0.7 ml/min, a solvent mixture consisting of 725 ml methanol, 275 ml water and 0.1 ml acetic acid. Authentic standard drugs are used to identify cis-, trans- and epi-LTB₄. The separation of the three isomers can be followed photometrically at the UV-maximum of 278 nm.

EVALUATION

The peak areas of cis-, trans- and epi-LTB₄ are measured as a function of drug concentration, and related to a control experiment without drug.

CRITICAL ASSESSMENT

The tested pathway involves the enzymatic steps of phospholipase A₂ and the 5-lipoxygenase. Thus inhibitors of these enzymes are to decrease formation of the three isomers of LTB₄. The step of phospholipase A₂ can be circumvented by the addition of exogenous arachidonic acid (1 µmol/l).

Inhibitors of LTA₄-hydrolase which catalyzes the intermediary conversion of LTA₄ to the biologically active cis-LTB₄, can also be identified by this assay. Such drugs are to exhibit an increased formation of the non-enzymatic hydrolysis products trans- and epi-LTB₄ at the expense of decreased cis-LTB₄.

MODIFICATIONS OF THE METHOD

Winkler et al. (1988) used differentiated U-937 cells expressing LTB₄ receptors to study Ca²⁺ mobilization in response to LTB₄.

Jones et al. (1995) tested inhibition of [³H]leukotriene D₄ specific binding in guinea pig lung, sheep lung, and dimethylsulfoxide-differentiated U837 cell plasma membrane preparations.

H.3.1.6.2**Formation of Lipoxygenase Products from
¹⁴C-Arachidonic Acid in Human Polymorphonuclear
Neutrophils (PMN) In Vitro****PROCEDURE**

PMN are prepared by the standard procedure of Böyum (1976). The first steps are carried out at room temperature: 50 ml citrated human blood are centrifuged at 130 g for 15 min. The pellet is resuspended in 20 ml Dulbecco's minimal essential medium, and subsequently underlayered with 15 ml lymphoprep. After centrifugation at 400 g for 25 min the pellet is resuspended in 28 ml Dulbecco's HBSS containing 3% dextran. After incubation at 0°C for 120 min the decanted supernatant is centrifuged at 400 g for 15 min. The pellet is resuspended in 1 ml Dulbecco's PBS, containing 11 µmol/l glucose, to a leukocyte count of 2 × 10¹⁰/l.

The method of HPLC determination of cellular metabolites of exogenous 1-C-14-arachidonic acid, as published by Borgeat and Samuelsson (1979), is modified as briefly described:

0.1 ml of the leukocyte suspension are incubated in Dulbecco's phosphate buffered saline (DPBS) at 37°C with the test drugs for 15 min. The incubation is then interrupted by cooling in an ice bath. Calcium ionophore A 23187 (final concentration 7 × 10⁻⁵ mol/l) and 1-C-14-arachidonic acid (final concentration 8.4 × 10⁻⁵ mol/l, 0.5 µCi) are added, and, after a second incubation period of 15 min at 37°C, terminated by the addition of 0.4 ml methanol. The assay mixture is then extracted with chloroform. The chloroform is evaporated, and, after redissolving the residue in a minor amount of methanol/water, analyzed by HPLC and radiomonitoring for the C-14-eicosanoids.

HPLC-conditions are as follows: Column: Nucleosil C-18, 5 µm; organic phase: 700 ml methanol, 300 ml water, 0.1 ml acetic acid; after 35 min change to pure methanol. Flow: 1 ml/min, 2000 psi. Radiomonitor: LB 507 (Berthold, Wildbad, FRG).

A viability assay (trypan blue exclusion) ascertains that cells remain intact during incubation periods.

EVALUATION

The radioactivity of the separated 5-HETE and LTB₄ is measured as a function of drug concentration, and related to a control experiment without drug. Two further lipoxygenase products, 12-HETE and 15-HETE, which are additionally generated under the test conditions, can be quantified in a similar way.

CRITICAL ASSESSMENT

The measured reaction sequence starts with arachidonic acid, and involves its transformation to 5-HETE by 5-lipoxygenase, as well as the subsequent enzymatic hydrolysis to LTB₄. Inhibitors of 5-lipoxygenase exhibit a decreased formation of 5-HETE and LTB₄. Effects of drugs on the side products 12-HETE and 15-HETE can also be studied in this test system.

H.3.1.6.3

Formation of Eicosanoids from ¹⁴C-Arachidonic Acid in Human Platelets In Vitro

PROCEDURE

Blood is drawn from the vena brachialis of healthy volunteers and collected into plastic tubes containing sodium citrate (0.38% final concentration (w/v)). After centrifugation at 100g for 15 min the platelet rich plasma (PRP) containing about 2.5×10^{11} platelets/l (Counter HT, Coulter Electronics, Krefeld, FRG) is saved, and kept at 20°C no longer than one hour until the experiment is started. Metabolism of ¹⁴C-arachidonic acid is followed by the HPLC procedures published by Weithmann et al. (1994), modifying the method of Powell (1985). PRP is mixed with the same volume of a citrate/D-glucose solution (27.35 g trisodium citrate · 2H₂O, 1.47 g citric acid and 27.74 g D(+)-glucose · H₂O ad 11 water) and centrifuged at 1000g for 20 min (4°C). The saved precipitate is re-suspended in Dulbecco's solution (DPBS, Serva, Heidelberg, FRG) in the original volume. 0.495 ml of this platelet suspension is incubated at 37°C for 15 min with the test compound. Subsequently eicosanoid formation is started by the addition of 5 µl of 1-¹⁴C-arachidonic acid solution (50 Ci/mol, 9×10^{-4} Ci/l, NEN, Dreieich, FRG). After 5 min (37°C) the reaction is stopped by adding 0.5 ml of chilled acetone/0.1 ml 1N HCl, cooled to 0°C, and extracted two times with 3 ml ethylacetate. The combined organic extracts are evaporated and the residue redissolved in 0.2 ml methanol. Aliquots are separated by

HPLC at a flow rate of 1.5 ml/min, using a C-18 Nucleosil column (5 µm, 125 × 4.6 mm, Bischoff, Leongang, FRG) connected with a pre-column C-18 Nucleosil (5 µm, 20 × 4.6 mm) of the same type. The formed ¹⁴C-eicosanoids are analyzed using a liquid scintillation flow detector LB 507 (Berthold, Wildbad, FRG). The radiochromatogram is analyzed by comparison with tritiated authentic eicosanoids (NEN, Dreieich, FRG). The elution system consists of the following solvent mixtures (elution time in parenthesis): I 725 ml water/275 ml acetonitrile/1 ml acetic acid (40 min), II 700 ml methanol/300 ml H₂O/1 ml acetic acid (40 min), III pure methanol (20 min).

EVALUATION

The radioactivity of the separated TXB₂ and PGE₂ is measured as a function of drug concentration, and related to a control experiment without drug addition.

A further lipoxygenase product, 12-HETE, which is additionally generated under the test conditions, can be quantified in a similar way.

Lasché and Larson determined PGI₂ by a bioassay based on its generation by aortic rings and assay by its ability to inhibit platelet aggregation.

CRITICAL ASSESSMENT

The measured reaction sequence starts with arachidonic acid, and involves its transformation to prostaglandin endoperoxides, which is catalyzed by cyclooxygenase. The unstable and short-living endoperoxides transform immediately into thromboxane A₂ by the action of thromboxane isomerase. TXA₂ is unstable, too, and yields the stable non-enzymatic hydrolysis product TXB₂.

Inhibitors of cyclooxygenase exhibit a decreased formation of TXB₂. Specific inhibition of the thromboxane isomerase step results in an accumulation of the mentioned endoperoxide intermediates, which due to their chemical instability are non-enzymatically transformed to the primary PGE₂. Thus, inhibitors of the TXA₂-isomerase lead to a significant increase of the PGE₂-peak at the expense of the TXB₂-peak. Effects of drugs on the side product 12-HETE can also be studied in this test system.

H.3.1.6.4

Stimulation of Inducible Prostaglandin Pathway in Human PMNL

PROCEDURE

The procedure of Herrmann et al. (1990) with the modification of Weithmann et al. (1994), is used to stimu-

late cyclohexamide-inhibitable generation of PGE₂ in human PMNL by LPS.

H.2.5 × 10⁹ PMNL/l culture medium (RPMI 1640, completed with 1 mmol/l sodium pyruvate, 5% FCS (w/v), 2 mmol/l glutamine and each 100 U/ml penicillin/streptomycin) are incubated with 100 μmol/l acetylsalicylic acid for 60 min (37°C, 5% CO₂), and the latter subsequently removed by four times washing with medium. 0.25 ml-aliquots (containing 2.5 × 10⁹ PMNL/l medium) are incubated with 0.1 mg/ml LPS (lipopolysaccharide from *Salmonella abortus equi*, Sigma GmbH, Deisenhofen, FRG) along with the test compound for 18 h (96-well plates, 37°C, 5% CO₂). The generation of eicosanoids is induced by the administration of 7 × 10⁻⁵ mol/l of the calcium ionophore A 23187. After incubation for 30 min at 37°C the plates are centrifuged at 400 g for 15 min. PGE₂ is determined in the supernatant, using an ELISA-kit commercially available from several distributors. Alternatively the test compound is added along with the calcium ionophore. At least 90% of the cells remain intact during incubation times (trypan blue exclusion assay).

EVALUATION

The PGE₂-concentration in the sample is determined from appropriate calibration curves. The PGE₂-concentration is measured as a function of drug concentration, and related to a control experiment without drug.

CRITICAL ASSESSMENT

Long term activation with LPS or other inflammatory effectors leads in human polymorphonuclear neutrophils to the stimulation of a prostaglandin synthesizing capacity, which under normal conditions is not present in this system. The described assay system detects compounds which interfere with this activation process.

Incubation of drugs with already activated cells allows to search for drugs that directly influence the enzyme activity.

H.3.1.6.5

COX-1 and COX-2 Inhibition

PURPOSE AND RATIONALE

Several assays were described to characterize COX-1 and COX-2-inhibitors, such as *in vitro* COX enzyme assay (Seibert et al. 1994), COX-2 protein extraction and analysis (Anderson et al. 1996), a human whole blood assay using LPS-induced PGE₂ production as an index for cellular COX-2 activity (Riendeau

et al. 1997) or whole-cell assays with transfected Chinese hamster ovary cells expressing COX-1 and COX-2 or COX-2 specific (osteosarcoma cells) and COX-1 specific (U937 cells) making use of PGE₂ production after arachidonic challenge as an index of cellular potency and selectivity of cyclooxygenase inhibitors (Chan et al. 1999; FitzGerald and Loll 2001; Rao et al. 2003).

PROCEDURE

In Vitro Cyclooxygenase Inhibition

The ability of test compounds to inhibit COX-1 and COX-2 (IC₅₀ values, μM) is determined using an enzyme immunoassay kit (Cyman Chemical, Ann Arbor, Mich., USA, no. 560101) (Uddin et al. 2004). This COX (ovine) inhibitor screening assay directly measures the amount of the prostaglandin PGF_{2α} produced in the cyclooxygenase reaction. The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds. Thus, this COX assay is more accurate and reliable than an assay based on peroxidase inhibition. The COX (ovine) inhibitor screening assay includes both ovine COX-1 and COX-2 enzymes in order to screen isozyme-specific inhibitors. This assay is an excellent tool, which can be used for general inhibitor screening, or to eliminate false-positive leads generated by less specific methods.

Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid to PGH₂. PGF_{2α} produced from PGH₂ by reduction with stannous chloride is measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of PG tracer is held constant while the concentration of PGs varies. This antibody-PG complex binds to a mouse anti-rabbit monoclonal antibody that was previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate for acetylcholinesterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation. Percent inhibition is calculated by the comparison of compound treated to various control incubations. The concentration of the

test compound causing 50% inhibition (IC_{50} , μM) is calculated from the concentration–inhibition response curve.

Inhibition Studies with Recombinant Human COX-1 and COX-2

Microsomal preparations of recombinant human COX-1 and COX-2 are prepared from a vaccine virus-COS-7 cell expression system (O'Neill et al. 1994). Recombinant human COX-1 and COX-2 are expressed in baculovirus-Sf9 cells, and enzymes are purified (Ouellet and Percival 1995; Cromlish and Kennedy 1996). Enzymatic activity is monitored continuously by either a fluorescence assay measuring the appearance of the oxidized form of the reducing agent cosubstrate homovanillic acid or by oxygen consumption (Ouellet and Percival 1995).

Classical non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors are time-dependent, irreversible inhibitors of hCOX-2, which is consistent with a two-step process involving an initial rapid equilibrium binding of enzyme and inhibitor, followed by the slow formation of a tightly bound enzyme–inhibitor complex. COX-2 inhibitors show a time-independent inhibition of hCOX-1, consistent with the formation of a reversible enzyme–inhibitor complex (Ouellet and Percival 1995; Riendeau et al. 2001).

HPLC Assay for Oxygenation of Radiolabeled Arachidonic Acid by COX-1

Purified recombinant human COX-1 (50 μl of 1 $\mu g/ml$ in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 μM hematin) is preincubated with 2 μl of the inhibitor solution (50 fold concentrated stock in DMSO, 0–2.5 mM) for 15 min. The reaction is then initiated by the addition of 5 μl of 1 μM [^{14}C]-arachidonic acid (0.005 μCi) to obtain a final concentration of 0.1 μM . After 7 min incubation at room temperature, the reaction is stopped by the addition of 5 μl 1 M HCl and 50 μl acetonitrile. Aliquots of 50 μl of each reaction mixture are analyzed for substrate conversion by reverse phase HPLC onto a C-18 Nova-Pak column (3.9 \times 150 mm) which is developed with acetonitrile/water/acetic acid (85:15:0.1) at 2 ml/min. Arachidonic acid metabolites and arachidonic acid eluted at 0.6–1 min and 2.2–2.5 min, respectively, are quantitated by a Packard radiochromatography detector. Percentages of inhibition are calculated from the difference in conversion of arachidonic acid to prostaglandin metabolites between inhibitor-treated samples and controls exposed to DMSO vehicle.

Determination of the Stoichiometry of Inhibitor Binding

Aliquots of purified COX-2 (0.25 mg/ml, concentration of subunit of 3.4 mM) are incubated in buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol) in the presence of various inhibitors (0–8 μM) for 15 or 30 min. An aliquot (20 μl) is then removed for determination of the cyclo-oxygenase activity which is monitored continuously by oxygen consumption by a Clark-type polarographic oxygen probe. The oxygen chamber is filled with 0.6 ml of reaction buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 μM hematin, 1 mM phenol, 100 μM arachidonic acid at 30° or 37°C) and the reaction is initiated by the addition of 20 μl of a solution of 4 mM hydrogen peroxide and 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) in assay buffer. Enzyme concentration is determined by amino acid concentration following acid hydrolysis.

Determination of the Dissociation Rate Constant of the Enzyme-Inhibitor Complex

Purified COX-2 (2.0 nmol, 2 ml) in 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 μM hematin, 0.1% β -octylglucoside is treated with 2.0 nmol [^{14}C]-DFU (18 Ci/mol) and incubated at 20°C for 3 h. A control (0.7 ml) is removed and 13 nmol unlabelled DFU is added to the remaining 1.3 ml of the mixture containing COX-2 and [^{14}C]-DFU. At timed intervals, 0.1 ml (in duplicate) is transferred to a Microcon-30 micro concentration device (Amicon) and the free inhibitor is separated from enzyme-bound inhibitor by centrifugation at 14,000 g for 6 min at 4°C. Buffer (0.1 ml) is added to the retentate and the centrifugation repeated. The filtrate and retentate are then removed and mixed with 10 ml scintillation fluid and counted in a liquid scintillation counter.

An aliquot of purified COX-2 (1.0 nmol) is treated with 1.25 mol equivalents of inhibitor or with DMSO vehicle control and incubated at 20°C for 1 h. The enzyme-inhibitor mixture is then transferred to a Pierce Microdialyzer 100 apparatus and dialyzed continuously against 2 l of buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM phenol, 0.1% octylglucoside) for 5 h at 22°C during which aliquots are moved and frozen at –70°C until assayed for cyclo-oxygenase activity by oxygen uptake as described above.

Recovery of Inhibitor from the COX-2-Inhibitor Complex

Purified COX-2 (0.79 nmol) is treated with 1.0 mol equivalent of inhibitor and the mixture is incubated for 60 min at room temperature. The remaining activity at this time is 4% that of a vehicle-treated con-

trol. The sample is then divided in two and the protein denatured by treatment with four volumes of ethyl acetate/methanol/1 M citric acid (30:4:1). After extraction and centrifugation (10,000 g for 5 min), the organic layer is removed and the extraction repeated. The two organic layers are combined and dried under N_2 . The extract is dissolved in 10 μ l of HPLC solvent mixture consisting of water/acetonitrile/acetic acid (50:41:0.1) and 50 μ l are injected onto a Novapak C-18 column (3.9 \times 150 mm) and developed at 1 ml/min. The inhibitor is detected by absorption at 260 nm and eluted with a retention time of 6.6 min in this system. Control experiments for inhibitor recovery are performed with incubation of the inhibitor in the absence of enzyme and processing of the samples in an identical fashion before quantitation by HPLC.

Spectrophotometric Assay of Recombinant Human COX-2

Enzymatic activity of the purified COX-2 is measured using a chromogenic assay based on the oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) during the reduction of PGG₂ to PGH₂ (Copeland et al. 1994). The assay mixture (180 μ l) contains 100 mM sodium phosphate, pH 6.5, 1 μ M hematin, 1 mg/ml gelatin, 2 to 5 μ g/ml of purified COX-2, and 4 μ l of the test compound in DMSO. The assay is also performed in the presence of the detergent Genapol X-100 at a final concentration of 2 mM. The mixture is preincubated at room temperature (22°C) for 15 min before the initiation of the enzymatic reaction by the addition of 20 μ l of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or hematin). For assays in the presence of Genapol, the arachidonic acid and TMPD solution is prepared in 50% aqueous ethanol. The enzyme activity is measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction as followed from the increase in absorbance at 610 nm. A low rate of non-enzymatic oxidation is observed in the absence of COX-2 and is subtracted before the calculation of the percentage of inhibition.

Whole-Cell Assays with Transfected Chinese Hamster (CHO) Cells Expressing COX-1 and COX-2

Stably transfected CHO cells expressing human COX-1 and COX-2 are cultured and assayed for the production of PGE₂ after stimulation with arachidonic acid (Kargman et al. 1996). Cells (0.3×10^6 cells in 200 μ l) are pre-incubated in HBSS containing 15 mM HEPES, pH 7.4, with 3 μ l of the test drug or DMSO vehicle for 15 min at 37°C before challenge with arachidonic acid. Cells are challenged for 15 min with an arachi-

donic acid solution (10% ethanol in HBSS) to yield final concentrations of 10 μ M arachidonic acid in the CHO[COX-2] assay and 0.5 μ M arachidonic acid in the CHO[COX-1] assay. In the absence of addition of exogenous arachidonic acid, levels of PGE₂ in samples from CHO[COX-1] are <30 pg PGE₂/10⁶ cells. In the presence of 0.5 μ M exogenous arachidonic acid, levels of PGE₂ in samples from CHO[COX-1] cells increase to 260 to 1500 pg PGE₂/10⁶ cells. After stimulation with 10 μ M exogenous arachidonic acid, levels of PGE₂ in samples from CHO[COX-2] cells increase from <120 to 700 to 1600 pg PGE₂/10⁶ cells. Compounds are tested in eight concentrations in duplicate using 3-fold serial dilutions in DMSO. COX activity in the absence of test compounds is determined as the difference in PGE₂ levels of cells challenged with arachidonic acid versus PGE₂ levels in cells mock-challenged with ethanol vehicle.

Arachidonic acid-dependent production of PGE₂ is measured in both cell lines after addition of test drugs. Indomethacin shows similar IC₅₀ values in both CHO[COX-1] and CHO[COX-2] cells, whereas specific COX-2 inhibitors show a 1,000-fold specificity.

Assays with Murine Macrophages

Mitchell et al. (1994), Hu et al. (2003), and Joo et al. (2004) used mouse peritoneal macrophages for evaluation of COX-2 inhibitors.

Adherent peritoneal macrophages were harvested from the peritoneal cells of male C5BL-6J mice after intraperitoneal injection of brewer thioglycollate medium (5 ml/100 g body weight) for 3 days. The peritoneal cells obtained from three to four mice were mixed and seeded in 48-well culture cluster at a cell density of 1×10^9 cells/l in RPMI-1640 supplemented with 5% newborn calf serum, penicillin, and streptomycin. After settlement for 2–3 h, non-adherent cells were washed by D-Hanks' balanced salt solution. Then macrophages were cultured in RPMI-1640 without serum. Almost all of the adherent cells were macrophages as assessed by Giemsa staining. Cell viability was examined by trypan blue dye exclusion. All incubation procedures were performed with 5% CO₂ in humidified air at 37°C.

COX-1 Assay

Macrophages were incubated with test compound at different concentrations or solvent (Me₂SO) for 1 h and were stimulated with calcimycin 1 μ mol/l for 1 h. The amount of 6-keto-PGF_{1 α} (a stable metabolite of PGI₂) in supernatants was measured by RIA according to manufacturer's guide. The inhibitory ratio was

calculated as

$$IR = \frac{(C_s - C_t)}{(C_s - C_c)}$$

C_s , C_t , C_c refer to 6-keto-PGF α concentration in supernatants of calcimycin, test compound, and control groups, respectively.

COX-2 Assay

Macrophages were incubated with test compound at different concentrations or solvent (Me₂SO) for 1 h and were stimulated with lipopolysaccharide (LPS) 1 mg/l for 9 h. The amount of PGE₂ in supernatants was measured by RIA. The inhibitory ratio was calculated using the same formula as in COX-1 assay section. C_s , C_t , C_c refer to PGE₂ concentration in supernatants of LPS, test compound, and control groups, respectively.

Statistical analysis data were expressed as the mean \pm SD of more than three independent experiments. Dose–inhibitory effect curves were fit through “up-hill dose–response curves, variable slope” using Prism, GraphPad version 3.00:

$$Y = \frac{1}{1 + 10^{[(\log IC_{50} - X) \times \text{Hillslope}]}}$$

Whole-Cell Assays with Osteosarcoma Cells (COX-2) and U937 Cells (COX-1)

The human osteosarcoma cell line has been shown to selectively express COX-2 by reverse transcription-polymerase chain reaction and immunoblot analysis, whereas undifferentiated human lymphoma U937 cells selectively express COX-1. The production of PGE₂ by these cells after arachidonic acid challenge is used as an index of cellular COX-2 and COX-1 activity, respectively. Test substances are preincubated for 5 to 15 min with the cells under serum-free conditions (HBSS) before a 10-min stimulation with 10 μ M arachidonic acid and measurement of PGE₂ production (Wong et al. 1997). COX activity in each cell line is defined as the difference in PGE₂ concentrations in samples incubated in the presence or absence of arachidonic acid.

Human Whole Blood Assay

For the COX-2 assay, fresh heparinized human whole blood is incubated with lipopolysaccharide from *E. coli* at 100 μ g/ml and with 2 μ l of vehicle or a test compound for 24 h at 37°C (Brideau et al. 1996). PGE₂ levels in the plasma are measured using radioimmunoassay after deproteination. For the COX-1 assay,

an aliquot of fresh blood is mixed with either DMSO or test compound and is allowed to clot for 1 h at 37°C. TBX₂ levels in the serum are measured using an enzyme immunoassay after deproteination.

MODIFICATIONS OF THE METHOD

Young et al. (1996) and Khanapure et al. (2003) used a similar assay to determine COX-1 and COX-2 enzyme activity in human whole blood. Human blood from non-fasted donors, who had not taken any aspirin or NSAIDs for 14 days, was collected in sodium heparin and distributed in 1-ml aliquots per well in a 24-well tissue culture plate. The plate was placed on a gently rotating platform shaker in a 5% CO₂ incubator at 37°C for 15 min. Test compounds were dissolved and diluted in dimethylsulfoxide (DMSO) and 1 μ l of each dilution of test compound was added per well in duplicate wells. To induce COX-2, lipopolysaccharide (LPS) from *E. coli* was added at 10 μ g/ml to appropriate wells 15 min after addition of the test compounds. For the stimulation of COX-1, the calcium ionophore A23187 was added to a final concentration of 25 μ M to separate wells 4.5 h after the addition of the test compounds. At 30 min after addition of A23187 or 5 h after LPS addition, all incubations were terminated by cooling on ice and adding EGTA to a final concentration of 2 mM. The blood samples were then transferred to 15 ml polypropylene centrifuge tubes and centrifuged at 1200 g for 10 min at 4°C. Then, 100 μ l of plasma was removed from each blood sample and added to 1 ml of methanol in a 15 ml polypropylene centrifuge tube, mixed vigorously, and stored overnight at –20°C. The next day, the samples were centrifuged at 2000 g for 10 min at 4°C, and the supernatants were transferred to glass tubes and evaporated to dryness. After reconstitution with EIA buffer, and appropriate dilution (2000-fold for COX-1 and 500-fold for COX-2), the samples were assayed for thromboxane B₂ using EIA kits (Cayman, Ann Arbor, Mich., USA) in duplicate wells.

Kalajdzic et al. (2002) showed that a preferential COX-2 inhibitor suppresses peroxisome proliferator-activated receptor induction of COX-2 gene expression in human synovial fibroblasts.

Berg et al. (1997) developed a cell assay system using the human erythroleukemic cell line HEL as a source for COX-1 and the human monocytic cell line Mono Mac 6 as a source for COX-2.

Kalgutkar et al. (2000) exploited biochemical differences between the COX isoforms to improve upon the selectivity of carboxylate-containing NSAIDs as COX-2 inhibitors.

Faust et al. (2003) recommended human peritoneal macrophages in culture as a model for studying inflammatory disorders *in vitro*.

Krause et al. (2003) reported that the NSAIDs indomethacin and diclofenac and a selective COX-2 inhibitor uncouple mitochondria in intact cells. To analyze the effects on energy metabolism of rat thymocytes, top-down elasticity analysis (Brand 1996, 1998; Ainscow and Brand 1999) was applied. Energy metabolism was conceptually divided into three blocks of reactions that generated and consumed the central intermediate mitochondrial membrane potential (ψ_m). The substrate oxidation subsystem encompassed all cellular catabolic reactions that provide the respiratory chain with its substrates NADH and succinate (e. g., glucose, fatty acid, and amino acid metabolism) and the electron transport chain itself, which together generate ψ_m . The ψ_m -consuming reactions were further divided into the subsystems proton leak and ATP turnover. The ATP turnover subsystem encompassed ATP synthesis by ATP synthase and subsequent ATP consumption by cellular pathways (e. g., ion pumps or protein synthesis). Manipulation of the biochemical properties of one block of reactions prompted a change of the intermediate ψ_m . The whole system evolved into a new steady state, which was determined by the kinetic responses of the other blocks to changes of ψ_m . Successive inhibition of one block of reactions permitted determination of these kinetic responses of the other blocks to changes of ψ_m . A comparison of the kinetic responses in the presence and absence of effectors (e. g., drugs) allowed for the identification of sites of actions.

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H.3.1.7 Influence of Cytokines

H.3.1.7.1 Induced Release of Cytokines (Interleukin-1 α , IL-1 β , IL-6, IL-8 and TNF α) from Human White Blood Cells In Vitro

PURPOSE AND RATIONALE

Cytokines represent a class of different, biologically highly potent peptides, that are endogenously syn-

thesized upon stimulation. They are involved in numerous cellular processes, such as inflammation, immunological responses and many others. Their broad pleiotrophic biological activities are best characterized by their former classification as

- B-cell activating and differentiating factor, endogenous pyrogen, osteoclast activating factor, thymocyte proliferation factor, monocyte cell factor, leukocyte endogenous factor (IL-1);
- hepatocyte stimulatory factor, hybridoma growth factor, haematopoietic cell stimulatory factor (IL-6);
- neutrophil chemotactic factor and adhesion inhibitor (IL-8);
- tumor necrosis factor (TNF α).

There are presently 18 cytokines with the name interleukin (IL) (Dinarello 2000). The concept of proinflammatory cytokines and antiinflammatory cytokines (IL-4, IL-10, IL-13) is fundamental to cytokine biology and novel drug discovery strategies.

Blocking IL-1 or TNF has been highly successful in patients with rheumatoid arthritis, inflammatory bowel disease, or graft-vs.-host disease.

The following procedure is used to detect compounds that interact with the lipopolysaccharide in ced cytokine release from human mononuclear blood cells. The cytokines measured are interleukines 1 α , 1 β , 6 and 8, as well as TNF α .

PROCEDURE

According to Böyum (1976) 10 ml of freshly prepared human citrated blood is diluted 1:1 with PM 16-buffer (Serva, Heidelberg, FRG), and underlayered with 15 ml Lymphoprep (Molter GmbH, Heidelberg, FRG), and subsequently centrifuged at 20°C with 400 g (Minifuge, Heraeus, Hanau, FRG) for 30 min. The cell fraction appearing as a white ring between the two phases is carefully removed by means of a syringe, diluted 1:1 (v/v) with PM 16 and again centrifuged for 15 min. The pellet is washed with 10 ml of RPMI 1640 (Gibco, Berlin, FRG), containing in addition 300 mg/l L-glutamine. The washed cell fraction is taken up in 1 ml RPMI 1640, containing in addition 300 mg/l L-glutamine, 25 mmol/l HEPES, 5% FCS and 100 IU/ml penicillin/streptomycin (Gibco). Using a cell counter (type IT, Coulter Diagnostics, Krefeld, FRG) the cell suspension which consists of about 90% lymphocytes and 10% monocytes is adjusted to approx. 5×10^9 cells/ml.

Synthesis and release of cytokines according to Tiku et al. (1986) is performed in 96 wells mi-

croter plates. To 0.23 ml of the cell fraction 500 ng LPS (lipopolysaccharide from *Salmonella abortus equii*, Sigma GmbH, Deisenhofen, FRG), dissolved in 0.01 ml dimethylsulfoxide/water (1:10, v/v), and the investigational drug, dissolved in 0.01 ml, are added. The cell suspension is now kept at 37°C/5% CO₂ in a common incubator. Incubation time is usually 20 h (for IL-6 and IL-8 only four or one hour, respectively). The reaction is stopped by placing the microtiter plate into an ice bath. The plate is then centrifuged at 2000 rpm for 2 min. The cytokine levels are determined in various aliquots of the supernatant using the appropriate ELISA-kit, which is commercially available from several distributors.

EVALUATION

The cytokine-concentration in the sample is determined from appropriate calibration curves. The cytokine-concentration is measured as a function of drug concentration, and related to a control experiment without drug. In general, Hostacortin (10 to 0,1 μ mol/l) is used as the standard compound.

CRITICAL ASSESSMENT

Drugs interfering with LPS-activation, biosynthesis and cellular release of cytokines are to exhibit activity in these assay systems. Usually cell viability is not altered, as assessed by the lactate-dehydrogenase test.

MODIFICATIONS OF THE METHOD

Van der Pouw-Kraan et al. (1992) examined the regulation of interleukin (IL)-4 production by human peripheral blood T cells. Production of IL-4 as measured by ELISA was shown to be regulated differently from IL-2 and INF- γ (also measured by ELISA).

A proinflammatory role for interleukin-18 (IL-18) in rheumatoid arthritis was attributed by Gracie et al. (1999).

The role of the interleukin-6 family of cytokines in inflammatory arthritis and bone turnover was reviewed by Wong et al. (2003).

Kim et al. (2005) reported an inhibitory effect of luteolin, a flavonoid from *Lonicera japonica*, on tumor-necrosis-factor- α -induced IL-8 production in human colon epithelia cells.

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cent anti-cytokine and anti-chemokine monoclonal antibodies are very useful for the intracellular staining and multiparameter flow cytometric analysis of individual cytokine-producing cells within mixed populations. Multicolor immunofluorescent staining with antibodies against intracellular cytokines and cell surface markers provides a high resolution method to identify the nature and frequency of cells which express particular cytokines (Sander et al. 1991, 1993; Jung 1993).

PROCEDURE

Cells and Cell Culture

Peripheral blood is obtained from healthy human volunteers. Mononuclear cells are isolated by Ficoll gradient centrifugation. For purification of T cells or further cell sorting, mononuclear cells are incubated with neuraminidase treated sheep red blood cells and centrifuged over Ficoll. Erythrocytes are eliminated by ammonium chloride lysis. For isolation of memory cells or naive cells, T cells are incubated with a cocktail of antibodies containing anti-CD16, anti-CD56, anti-CD20, anti-CD14, (anti-CD8), and anti-CD45RA or anti-CD45R0. Cell sorting is done with the magnetic cell sorter (MACS) according to Abts et al. (1989), Miltenyi et al. (1990) using rat anti-mouse IgG1 or Ig2a antibodies labeled with superparamagnetic beads (Miltenyi, Bergisch Gladbach, Germany). Depleted cells are highly enriched with CD4⁺(CD3⁺)CD45R0⁺ or CD4⁺(CD3⁺)CD45R0⁻CD45RA⁺ cells (>95%) and are referred to as memory cells and naive cells (CD4⁺CD45R0⁻CD45RA⁺) respectively. Only depleted cells are used for experiments.

Cells (2×10^5 /100 ml) are cultured in 96 well flat bottom plates for various periods of time at 37°C and 8% CO₂ in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2×10^{-5} M mercaptoethanol and 10% AB serum. Cells are stimulated with phorbol 12-myristate 13-acetate 1–10 ng/ml+1 µM ionomycin, phytohemagglutinin 2.4 µg/ml or phytohemagglutinin 2.4 µg/ml+phorbol 12-myristate 13-acetate 1 ng/ml in the presence or absence of 3 µM monensin (Sigma).

H.3.1.7.2

Flow Cytometric Analysis of Intracellular Cytokines

PURPOSE AND RATIONALE

Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters, including size and granularity, as well as the expression of surface and intracellular markers defined by fluorescent antibodies. Fluores-

Staining

Cultured cells are washed twice in Hanks' balanced solution (HBSS) and then fixed in ice-cold HBSS containing 4% paraformaldehyde for 10 min. After two further washes in HBSS the cells are resuspended to 2×10^5 in 300 µl HBSS containing 0.1% saponin, 10% AB serum, 100 µg/ml goat IgG and 0.01 M HEPES buffer. After 10 min, the cells are spun

down and cytokine specific antibodies diluted in HBSS with 0.1% saponin and 0.1 M HEPES buffer (saponin buffer) are added at a concentration of 1 µg/ml for 30 min at room temperature. Cells are washed twice in saponin buffer and subsequently incubated with isotype-specific second step antibodies in a concentration ranging from 0.5 to 5 µg/ml for 20 min in the dark. Cells are washed in saponin buffer and stained with streptavidin conjugates or in the case of surface staining incubated with 200 µg/ml mouse IgG diluted in saponin buffer for 15 min. After subsequent washing in saponin buffer cells are washed twice in HBSS and stained for 20 min with different antibodies in order to determine their surface phenotype. As a last step, cells are washed in HBSS.

Flow Cytometry

A FACScan flow cytometer (Becton Dickinson, Mountain View, USA) equipped with a 15 mW argon ion laser and filter settings for fluorescein-isothiocyanate (530 nm), phycoerythrin (585 nm) and TRICOLOR (Medac, Hamburg, Germany) or PerCP (Becton Dickinson, USA) emitting in the deep red (>650 nm) is used.

EVALUATION

5000–10000 cells are computed in list mode and analyzed using the FACScan research software (Becton Dickinson)

MODIFICATIONS OF THE METHOD

Slauson et al. (1999) combined the analytical power of flow cytometry with mitogen-driven, whole blood lymphocyte activation and proliferation assays to investigate the *in vitro* mechanism of action of malononitrilamides.

Protocols for immunofluorescent staining of intracellular cytokines for flow cytometric analysis are provided by BD Phar Mingen, Life Science Research Europe, Heidelberg, Germany.

Ashcroft and Lopez (2000) highlighted the opportunities in high throughput flow cytometry (HTFC) which are opened by commercial high speed machines. The specifications of these machines are cell analysis rates over 100,000 cells/s and cell sorting rates of 55,000 cells/s with high purity.

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H.3.1.7.3

Screening for Interleukin-1 Antagonists

PURPOSE AND RATIONALE

Interleukin-1 α and -1 β are potent regulators of inflammatory processes. The naturally occurring IL-1 receptor antagonist (IL-1ra) is effective *in vitro* and *in vivo* in modulating biological responses to IL-1 (Carter et al. 1990; Hannum et al. 1990; Schreuder et al. 1995). Using a combination of anion exchange, gel filtration, and reverse-phase HPLC, three species of native IL-1ra were identified. An unglycosylated, intracellular isoform is designated as icIL-1ra (Lennard 1995; Arend et al. 1991, 1998).

A cell-free, non-isotopic assay has been developed to discover molecules that compete with the natural ligands for binding to the active sites of the type-I IL-1 receptor. The key reagents are the IL-1 receptor antagonist, a recombinant soluble form of the receptor (sIL-1R), and a specific anti-sIL-1R non-neutralizing monoclonal antibody (Sarrubi et al. 1996).

PROCEDURE

Proteins

The extracellular portion of the type-I IL-1 receptor (sIL-1R) is expressed on the membrane of Chinese hamster ovary cells using a phosphatidylinositol-glycan linkage (PIG-tail). Its expression, cleavage with phosphoinositol-specific phospholipase C, and purification are performed according to Whitehorn et al. (1995).

The three IL-1 ligands are expressed in *Escherichia coli* using synthetic genes (Dower et al. 1989; Yanofsky and Zurawski 1990). The purification of IL-1ra

and IL- β is accomplished according to Schreuder et al. (1995) and Yem et al. (1988).

IL-1 α is purified as follows: *E. coli* cell sonicates are precipitated with 2 M ammonium sulfate, and the pellet is resuspended in TE (25 mM Tris/HCl, pH 8.9, 1 mM EDTA), dialyzed against the same buffer and loaded on a DEAE-Sephacryl column equilibrated with TE. The protein is eluted with a linear NaCl gradient to 300 mM. Ammonium sulfate to 0.8 M is added to the IL-1 α -containing fractions which are loaded onto a phenyl-Sephacryl column equilibrated with TE containing 0.8 M ammonium sulfate. The elution is performed with a linear gradient to TE with no salt. IL-1 α -containing fractions are concentrated and chromatographed on a Sephacryl S-200 column in PBS (phosphate-buffered saline: 20 mM sodium phosphate, pH 7.3, 150 mM sodium chloride).

Fluorescein-labeled IL-1 α is obtained by incubating 1 mg/ml IL-1 α with 1 mg/ml fluorescein isothiocyanate in PBS for 2 h at room temperature in the dark. The reaction solution is passed directly over a G-25 column (Pharmacia) equilibrated with PBS to remove unreacted fluorescein isothiocyanate.

The monoclonal antibody Mab79 is used as direct dilutions ($1:10^5$ – 10^6) of ascitic fluid in PBSA (PBS containing 0.3% bovine serum albumin). Horseradish-peroxidase-linked anti-mouse IgG polyclonal antibody is used.

Protein concentrations are determined using the Bio-Rad protein assay kit, based on the dye-binding procedure according to Bradford (1976). BSA is used as reference protein.

Immobilized-Ligand IL-1 Receptor Binding Assay

Essentially the same procedure can be used for both manual and automated versions of the assay, with all steps and incubations performed at room temperature. In the automated assay a Beckman Biomek 1000 Work-Station was used for all steps, from coating to spectrophotometric measurements.

Ligand immobilization is obtained by incubation of 3.6 μ g/ml IL-1ra in 50 μ l PBS in flat-bottomed culture-treated microplate wells, equivalent to 10 pmol/well of IL-1ra. After overnight incubation microplates are emptied and 250 μ l/well of 3% BSA in PBS is added to block unreacted sites. After 2 h of incubation and three washes with an excess of PBS, the ligand-coated microplates are ready for the receptor binding reaction.

In separate microplates with U-shaped wells, 12 μ l of samples (containing up to 50% DMSO or DMF) or controls (the same solution without compound) is

mixed with 48 μ l of 150 pM sIL-1R in PBSA. Then 50 μ l of these mixtures is transferred to the IL-1ra-coated plates (equivalent to 6 fmol/well of sIL-1R) and incubated for 2 h. Microplates are then washed twice with PBS and 50 μ l of 1:500,000 dilution of Mab79 ascitic fluid in PBSA is added to each well. After 1 h of incubation, 25 μ l of 1:100 dilution of HRP-labeled anti-mouse IgG in PBSA is added and the incubation prolonged for an additional hour. Plates are finally washed four times with PBS and bound peroxidase activity is measured spectrophotometrically, using either *o*-phenylenediamine (OPD) or tetramethylbenzidine (TMB) as substrate. In the first case, 150 μ l of 1 mg/ml OPD in 0.1 M citric acid, pH 5.0, containing 0.03% of a 35% solution of hydrogen peroxide is added and, after color development, the reaction is stopped with 50 μ l of 4.5 M sulfuric acid. Alternatively, 100 μ l of 0.1 mg/ml TMB in 25 mM citric acid and 50 mM sodium phosphate, containing 0.02% hydrogen peroxide (35% solution), is added and the reaction is stopped with 50 ml of 2.5 M sulfuric acid.

EVALUATION

Absorbance (at 492 nm for OPD and 450 nm for TMB) is measured using either a Titertek microplate reader (for the manual procedure) or directly by the Biomek 1000 WorkStation (in the automated version). IC₅₀ values can be calculated from dose–response curves.

CRITICAL ASSESSMENT OF THE METHOD

Since no cells or cell membranes are used, the assay is very robust, with no interference from membrane-perturbing agents, and has high resistance to the organic solvent normally used to resuspend compounds of chemical libraries.

MODIFICATIONS OF THE METHOD

High-affinity type I interleukin-1 receptor antagonists were discovered by screening recombinant peptide libraries (Yanosky et al. 1996).

Akeson et al. (1996a) developed an *ex vivo* method for studying inflammation in cynomolgus monkeys using whole blood for analysis of IL-1 antagonists administered *in vivo*. Animals were given an i.v. infusion of IL-1ra, and blood samples were taken pre-infusion and during the infusion. The samples were incubated with or without IL-1 β and the subsequent *ex vivo* induction of IL-6 determined. This allows the analysis of the *in vivo* efficacy of antagonists without exposing the animals to IL-1.

A novel low-molecular-weight antagonist, selectively binding the type I IL-1 receptor and blocking the *in vivo* responses to IL-1 was described by Akeson et al. (1996b).

Evaluation of the IL-1 receptor antagonist IL-1ra in a rodent abscess model of host resistance was published by Colagiovanni and Shopp (1996).

Blocking monoclonal antibodies (mAbs) specific to mouse IL-1 receptor antagonist (IL-1ra) were prepared by immunizing Armenian hamsters with recombinant mouse IL-1ra by Fujioka et al. (1995). A sensitive and specific ELISA against mouse IL-1ra was established.

Miesel et al. (1995) tested the anti-arthritic reactivity of the IL-1 receptor antagonist IL-1ra in male DBA/1xB10A(4R) mice with arthritis induced by intraplantar injection of potassium peroxochromate. Then 3 μ mol/kg K₃CrO₈ was administered topically into the left hind paws and 1 h after the induction of arthritis, 2 mg/kg IL-1ra was administered intraperitoneally, which was repeated on day 2. An arthritis index was determined daily.

Nakae et al. (2003) found that IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist.

Redlich et al. (2003) reviewed rheumatoid arthritis therapy after tumor necrosis factor and interleukin-1 blockade.

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H.3.1.7.4

Inhibition of Interleukin-1 β Converting Enzyme (ICE)

PURPOSE AND RATIONALE

Programmed cell death (apoptosis) is effected through a cascade of intracellular proteases known as caspases (Alnemri et al. 1996). The interleukin-1 β -converting enzyme (ICE), alternatively known as caspase-1, was

the first such protein identified on the basis of its sequence homology to the pro-apoptotic *Caenorhabditis elegans* gene product, ced-3 (Yuan et al. 1993). The caspase family includes 10 reported human homologs of ICE. By sequence homology comparisons between three caspase subfamilies have been identified. The ICE subfamily includes three caspases: ICE, TX (caspase-4), and TY (caspase-5). The CPP32 subfamily includes CPP32 (caspase-3), CMH-1 (caspase-7), and MCH-2 (caspase-6). A third caspase subfamily includes ICH-1 (caspase-2), FLICE (caspase-8) and caspases-9 and -10.

ICE processes pro-IL-1 β to yield active IL-1 β , which plays a pivotal role in inflammatory cell activation (Dinarello 1996) and is known to inhibit the expression of apoptosis (Tatsuda et al. 1996). Inhibition of IL-1 β formation is an approach for the treatment of inflammatory disorders such as rheumatoid arthritis. Livingstone (1997) presented a review on *in vitro* and *in vivo* studies of peptidyl ICE inhibitors.

PROCEDURE

Neutrophil Isolation

Neutrophils are isolated from healthy volunteers by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient (Lee et al. 1993). Isolated neutrophils are resuspended in polypropylene tubes at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin solution. Neutrophil purity is assessed by size and granularity on flow cytometry.

Quantification of Apoptosis

Neutrophil apoptosis is quantified by flow cytometry as the percentage of cells with hypodiploid DNA (Nicoletti et al. 1991). Cells are centrifuged at 200 g for 10 min, gently resuspended in 500 μ l of hypotonic fluorochrome solution (50 μ g/ml propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, and 0.1% Triton X-100) and stored in the dark at 4°C for 3–4 h before analysis using a Coulter Epics XL-MCL cytofluorometer. A minimum of 5000 events are collected and analyzed. Apoptotic nuclei are distinguished from normal neutrophil nuclei by their hypodiploid DNA; neutrophil debris is excluded from analysis by raising the forward threshold. Apoptotic nuclei appear as a broad hypodiploid DNA peak which is easily discernible from the narrow peak of cells with normal diploid DNA content. Apoptosis is assessed at 24 h after treatment.

Assay of Caspase-1 Activity

Cell lysates are prepared from the membrane fraction of 20×10^6 neutrophils following experimental manipulation. Aliquots of the lysates (10 μ l) are diluted in assay buffer 100 mM HEPES (pH 7.4), 10% sucrose, and 0.1% 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate) containing 20 μ M Ac-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Calbiochem) and then incubated for 45 min at room temperature. The release of 7-amino-4-methylcoumarin is detected by continuous measurement using a Perkin-Elmer LS50 luminescence spectrometer with an excitation of 380 nm and an emission slit at 460 nm. Specific ICE (caspase-1) activity is measured as pmol/s per milligram of protein.

EVALUATION

Individual experiments are repeated a minimum of four times; results are expressed as the mean \pm SD. Analysis is performed using the Student's *t*-test or ANOVA with Scheffé's correction.

MODIFICATIONS OF THE METHOD

Norman et al. (1997) found that the severity and mortality of experimental pancreatitis are dependent on interleukin-1 converting enzyme.

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H.3.1.7.5**Nuclear Factor- κ B****H.3.1.7.5.1****General Considerations**

Nuclear factor kappa B (NF- κ B) is an inducible transcription factor of the Rel family, sequestered in the cytoplasm by the I κ B family of proteins. NF- κ B exists in several dimeric forms, but the p50/p65 heterodimer is the predominant one. Activation of NF- κ B by a range of physical, chemical, and biological stimuli leads to phosphorylation and proteasome dependent degradation of I κ B, leading to the release of free NF- κ B. This free NF- κ B then binds to its target site (κ B sites in the DNA), to initiate transcription. This transcription is involved in a number of diseases including cancer, AIDS, autoimmune diseases, and inflammatory disorders. The nuclear factor κ B is essential for the transcriptional regulation of the proinflammatory cytokines IL-1, IL-6, IL-8 and tumor necrosis factor α . NF- κ B is also the target for glucocorticoid-mediated IL-8 repression. Reduction-oxidation (redox) regulation is implicated in the activation of NF- κ B. (Mukaida et al. 1994, Aupperle et al. 1999, Christman et al. 2000a, 2000b, Nichols et al. 2001, D'Acquisto et al. 2002, Nishi et al. 2002, Palanki 2002, Tian et al. 2002, Heynink et al. 2003, Aggarwal et al. 2004, Pande and Ramos 2003, 2005, Kaltschmidt et al. 2005). The IKK complex, as a critical activator of NF- κ B function, consists of a core of three subunits, two of which, namely IKK α and IKK β contain functional kinase domains and are capable of phosphorylating I κ B at specific N-terminal residues to initiate its ubiquitination. In contrast, the third core subunit of the IKK complex, called NEMO (also known as IKK γ or IKKAP) is a non-catalytic component that functions as a key regulator of IKK activity (Gosh and Karin 2002, Karin et al. 2004).

Studying joint erosion in rheumatoid arthritis O'Gradaigh et al. (2003) found that interactions between tumor necrosis factor α , interleukin, and receptor activator of nuclear factor κ B ligand (RANKL) regulate osteoclasts

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H.3.1.7.5.2**Inhibition of Nuclear Factor- κ B****PURPOSE AND RATIONALE**

Several authors studied the inhibition of NF- κ B by compounds.

Staal et al. (1990) found that intracellular thiols regulate activation of nuclear factor κ B and transcription of human immunodeficiency virus. Schrenk et al. (1992) reported dithiocarbamates as potent inhibitors of nuclear factor κ B activation in intact cells. Natara-

jan et al. (1996) described caffeic acid phenyl ester as a potent and specific inhibitor of activation of nuclear transcription factor NF- κ B. Geng et al. (1997) reported that S-allyl cysteine inhibits activation of nuclear factor κ B in human T cells. Hiramoto et al. (1998) described nuclear-targeted suppression of NF- κ B by a quinone derivative. Ichiyama et al. (1999) found inhibition of peripheral NF- κ B activation by a central action of α -melanocyte-stimulating hormone. Castrillo et al. (2001) described inhibition of the nuclear factor κ B pathway by tetracyclic kaurene diterpenes in macrophages with specific effects on NF- κ B-inducing kinase activity and on the coordinated activation of ERK and p38 MAPK. Kang et al. (2001) reported that genistein prevents nuclear factor kappa B activation and acute lung injury induced by intratracheal treatment of rats with lipopolysaccharide. Lee et al. (2002) found that kamebakaurin, a kaurane diterpene, inhibits NF- κ B by directly targeting the DNA-binding activity of p50 and blocks the expression of antiapoptotic NF- κ B target genes. Palanki et al. (2002) reported structure–activity relationship studies of ethyl 2-[3-methyl-2,5-dioxo(3-pyrrolyl) amino]-4-(trifluoromethyl)pyrimidine-5-carboxylate, an inhibitor of AP-1 and NF- κ B mediated gene expression. Kim et al. (2004) found that tripolide, a natural compound extracted from the Chinese herb *Tripterygium wilfordii*, inhibits murine-inducible nitric synthase expression by down-regulating lipopolysaccharide-induced activity of nuclear factor κ B and c-Jun NH₂-terminal kinase. Jancso et al. (2005) studied the effect of acetylsalicylic acid on nuclear factor- κ B activation and on late preconditioning against infarction of the myocardium. Kunsch et al. (2005) described redox-sensitive inflammatory gene expression of AGIX-4207, an antioxidant and anti-inflammatory compound.

Tse et al. (2005) found that honokiol, a small molecular weight lignan isolated from *Magnolia officinalis*, inhibits tumor-necrosis-factor- α -stimulated NF- κ B activation and NF- κ B-regulated gene expression through suppression of inhibitor κ B kinase (IKK) activation.

PROCEDURE

Honokiol was dissolved in DMSO as a 100 mM stock solution and stored at -20°C.

Cell Culture

The cell lines used in this experiment were obtained from American Type Culture Collection (Manassas, Va., USA). U937 and HL-60 cells were grown

in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco, NY, USA) at 37°C in humidified 5% CO₂ atmosphere. MCF-7 and HeLa cells were cultured in Eagles' minimum essential medium containing 10% fetal bovine serum under the same condition.

Electrophoretic Mobility Shift Assay (EMSA)

For the electrophoretic mobility shift assay according to Chaturvedi et al. (2000), equal quantities of nuclear protein (5 μ g) from each sample was incubated with radiolabeled gel shift oligonucleotides for 15 min at 37°C and then resolved on a non-denaturing 5% (w/v) polyacrylamide gel. The gel was dried onto 3 MM blotting paper and used to expose X-ray film for overnight at -70°C. For supershift assays, 1 μ l of antiserum recognizing each of the NF- κ B subunits was added to the EMSA reaction 30 min before electrophoresis.

Western Blot Analysis

To obtain the whole-cell lysates, samples containing 1×10^7 cells were pelleted, washed twice with ice-cold PBS, then lysed in 150 μ l of modified RIPA buffer [50 mM Tris-Cl, 1% (v/v) NP-40, 0.35% (w/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.4] supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na₃VO₄, 10 μ g/ml each of aprotinin, leupeptin and pepstatin A for 20 min at 4°C. Supernatants after centrifugation at 14,000g for 15 min at 4°C were collected. Alternatively, cytoplasmic extracts were prepared. Samples containing 30–50 μ g of protein were separated on SDS-polyacrylamide gel and then transferred onto nitrocellulose membrane (0.45 μ m, Bio-Rad). Membranes were immunoblotted with primary antibodies and followed by horseradish-peroxidase-conjugated secondary antibodies (1:5000) and visualized by ECL (Amersham Biosciences) according to manufacturer's instructions.

IKK Assay

Whole-cell lysates (500 mg) were collected in modified RIPA buffer without sodium deoxycholate, and cellular debris was removed by high-speed centrifugation. Lysates were pre-cleared by incubation with 0.25 μ g of the appropriate control IgG together with 20 μ l of protein A/G plus (25%, v/v) agarose conjugate for 30 min at 4°C, followed by centrifugation. Supernatants were then incubated with 1 μ g of anti-IKK α/β for 2 h at 4°C, and then 20 μ l of protein A/G plus agarose was added and incubated at 4°C on a rocker platform

overnight. After several washes with IP buffer and PBS, beads containing IKK α/β were incubated with 0.5 μg GST- I κ B α substrate, 200 μM ATP in 20 μl kinase buffer (50 mM Tris-Cl pH 7.4, 20 mM MgCl₂, 20 mM β -glycerophosphate, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 0.5 mM DTT, 1 mM benzamidine, 10 $\mu\text{g}/\text{ml}$ aprotinin and 1 $\mu\text{g}/\text{ml}$ leupeptin) at 30°C for 30 min. Kinase reactions were stopped by the addition of 5 μl 5 \times Laemli's loading buffer and heated at 100°C for 5 min. The samples were resolved by 8% SDS-PAGE, electro-transferred to nitrocellulose membrane and probed with anti-phosphor-I κ B α (Ser³²) antibody (1:1000). Membranes were re-probed with anti-IKK to ensure equal loading and the presence of total IKK protein.

Plasmids, Transfection and NF- κ B-Dependent-Luciferase Reporter Assay

To measure the effect of honokiol on TNF- α -induced NF- κ B-dependent gene reporter transcription, HeLa cells were seeded into 24-well plates at a density 1.6×10^5 cells/well for 24 h. Subsequently, cells were transiently transfected with p3EnhConA-Luc or pControl-Luc (0.75 μg) using LipofectAMINE 2000 (Invitrogen). To normalize the transfection efficiency, cells were co-transfected with 0.25 μg of β -galactosidase control vector. After overnight incubation, cells were pre-treated with honokiol for 12 h following by 5 ng/ml TNF- α for 15 h and then harvested with 1 \times reporter lysis buffer (Promega, Madison, Wis., USA). Relative luciferase activity was measured with a Bright-GLO luciferase assay system using POLARStar OPTIMA luminometer (BMG Labtechnologies). Luciferase activity was normalized with β -galactosidase activity, as measured by the Beta-GLO luciferase assay system according to the manufacturer's instructions.

To measure the effect of honokiol on NF- κ B-dependent gene reporter transcription induced by various kinases, HeLa cells were transfected with p3EnhConA-Luc and β -galactosidase control vector together with 0.2 μg of expression vectors. After 5 h of incubation, cells were treated with honokiol for 24 h and then harvested and assayed as described above.

EVALUATION

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Two compounds (A and B) were considered enhancing each other's actions if the effect of combined treatment (AB) was larger than the sum of their individual effects ($AB > A + B$) after subtraction of the respective background control values.

MODIFICATIONS OF THE METHOD

Mortellaro et al. (1999) reported that the immunosuppressive drug PNU156804 blocks IL-2-dependent proliferation and NF- κ B and AP-1 activation in human primary T lymphocytes.

Spencer et al. (1999) found in murine NIH3T3 fibroblasts and primate COS-7 cells that taxol selectively blocks microtubule-dependent NF- κ B activation by phorbol ester via inhibition of I κ B α phosphorylation and degradation.

Yan and Polk (1999) reported that aminosalicyclic acid inhibits I κ B kinase α phosphorylation of I κ B α in mouse intestinal epithelial cells.

Acarin et al. (2000) found that oral administration of the anti-inflammatory substance triflusal results in the downregulation of constitutive transcription factor NF- κ B in the postnatal rat brain.

Eberhardt et al. (2002) studied involvement of nuclear factor κ B and Ets transcription factors in glucocorticoid-mediated suppression of cytokine-induced matrix metalloproteinase-9 expression in rat mesangial cells.

Kang et al. (2002) showed that inhaled nitric oxide attenuated acute lipopolysaccharide-induced lung injury in rabbits via inhibition of nuclear factor- κ B.

Macotela et al. (2002) found on rat pulmonary fibroblasts that 16K prolactin induces NF- κ B activation.

Roshak et al. (2002) reported small-molecule inhibitors of NF- κ B for the treatment of inflammatory joint disease.

Burke et al. (2003) found that BMS-345541 is a highly selective inhibitor of I κ B kinase that binds at an allosteric site of the enzyme and blocks NF- κ B-dependent transcription in mice.

Castro et al. (2003) described β -carboline as inhibitors of the NF- κ B kinase.

Clarke et al. (2003) reported that two distinct phases of virus-induced nuclear factor κ B regulation enhance tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in virus-infected cells.

Murata et al. (2003) described discovery of selective IKK- β serine-threonine protein kinase inhibitors.

Yadav et al. (2003) reported that a diarylheptanoid from lesser galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, P44/42, and transcription factor nuclear factor- κ B.

HMG-CoA reductase inhibitors (statins) inhibited the binding of nuclear proteins to both NF- κ B and AP-1 DNA consensus oligonucleotides in human endothelial and vascular smooth muscle cells as assayed by EMSA (Dichtl et al. 2003).

Gupta et al. (2004) discussed the essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor *kappa*B and induction of apoptosis.

Mühlbauer et al. (2004) studied differential effects of deoxycholic acid and taurodeoxycholic acid on NF- κ B signal transduction and IL-8 gene expression in human colonic epithelial cells.

Syrovets et al. (2005) found that acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF- α induction by direct interaction with I κ B kinases.

Eberhardt et al. (2005) found that dissociated glucocorticoids equipotently inhibit cytokine- and cAMP-induced matrix degrading proteases in rat mesangial cells.

Matsubara et al. (2005) found that a histamine H1 receptor antagonist blocks histamine-induced proinflammatory cytokine production through inhibition of Ca²⁺-dependent protein kinase C, Raf/MEK/ERK and IKK/ I κ B/NF- κ B signal cascades.

Mendoza-Milla et al. (2005) reported that NF- κ B activation but not PIK/Akt is required for dexamethasone-dependent protection against TNF- α cytotoxicity in L929 cells.

APPENDIX

Electrophoretic Mobility Shift Assay (EMSA)

Most papers mentioned above used the electrophoretic mobility shift assay (EMSA) for determination of nuclear factor κ B. This method is one of the most sensitive ones for studying the DNA-binding properties of a protein. It can be used to deduce the binding parameters and relative affinities of a protein for one or more DNA sites or for comparing the affinities of different proteins to the same sites (Fried 1989). It is also useful for studying higher-order complexes containing several proteins, observed as a “supershift assay.” EMSA can also be used to study protein- or sequence-dependent DNA bending (Crothers et al. 1991).

In an EMSA, or simple “gel shift,” a ³²P-labeled DNA fragment containing a specific DNA site is incubated with a candidate DNA-binding protein. The protein–DNA complexes are separated from free (unbound) DNA by electrophoresis through a non-denaturing polyacrylamide gel. The protein retards the mobility of the DNA fragments to which it binds; thus, the free DNA migrates faster through the gel than does the DNA–protein complex. An image of the gel reveals the positions of the free and bound ³²P-labeled DNA.

A protocol of the detailed procedure was described by Carey and Smale (2000).

PROCEDURE

1. Prepare a 40-ml 4.5% native acrylamide gel (using 1- to 1.5-mm spacers)

| | |
|------------------------------------------------------------------------------|-------------|
| Acrylamide mix (30%:29:1 acrylamide:bisacrylamide) | 6 ml |
| 5 × Tris-borate/EDTA (TBE) buffer | 4 ml |
| Glycerol (20% vol/vol) | 2 ml |
| Water | 28 ml |
| Ammonium persulfate (10% solution in water) | 300 μ l |
| <i>N,N,N,N</i> -tetramethylethylenediamine (add just before pouring the gel) | 30 μ l |

Pre-run the gel for 2 h at 10 mA.

2. Set up binding reactions in 0.5-ml siliconized microcentrifuge tube

| | |
|-------------------------------------------------------|---------------|
| Recombinant protein (0.5–100 ng) | 1.00 μ l |
| ³² P-labeled DNA template (ideally 1 fmol) | 1.00 μ l |
| Poly(dI:dC) (1 μ g/ μ l) | 0.20 μ l |
| Dimethylsulfoxide (0.1 M) | 0.10 μ l |
| MgCl ₂ (0.1 M) | 0.75 μ l |
| Buffer D | 6.70 μ l |
| Water | to 10 μ l |

Buffer D is 20 mM HEPES-KOH (pH 7.9), 20% glycerol (vol/vol), 0.2 mM EDTA, 0.1 M KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT. Add PMSF and DTT just before use.

3. Incubate the reaction at 30°C (incubate at 15°C–25°C or on ice for crude extracts) for 1 h.
4. Load the samples directly (with no dye) onto gel. Carefully layer the mix onto the bottom of the well and observe the schlieren line form at the glycerol–buffer interface.
5. Run the gel for desired time at 10 mA; for a 30-bp fragment, allow the bromophenol blue dye to migrate about two-thirds of the way down the gel.
6. When the electrophoresis run is complete, carefully pour out the buffer into the sink and remove the gel from the apparatus. Remove the comb and split the plates, leaving the gel attached to one plate.
7. (Optional) Fix the gel in gel fixing solution (200 ml methanol, 100 ml acetic acid, 700 ml water) at 15°C–25°C for 15 min.
8. Place the gel on two sheets of Whatman 3MM paper. Cover the other side of the gel with Saran Wrap and dry on a gel dryer at 70°C for 1 h.
9. Expose the gel to autoradiography film or to phosphorimager screen overnight.

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H.3.1.8

TNF- α Antagonism

H.3.1.8.1

General Considerations

TNF- α has been cloned and identified by Beutler et al. (1985). It is primarily produced in macrophages, lymphocytes, neutrophils, endothelial cells, keratinocytes and fibroblasts during acute inflammatory reactions. TNF- α is a member of a large family of proteins and receptors that are involved in immune regulation such as kinases, including nuclear factor kappa B (NF- κ B), p38 MAP kinase, JUN kinases and others. Therefore, it is a therapeutic target for immune-mediated inflammatory diseases (Pfizenmaier et al. 1996; Van Deventer 1997; Rath and Aggarwal 1999; Feldmann et al. 2001; Furst et al. 2001; Taylor 2001; Doggrell 2002; Braun et al. 2003; Sharma and Anker 2003; Louie et al. 2003; Nanes 2003; Peng et al. 2003; Chen et al. 2003; Tay-

lor et al. 2004; Gupta et al. 2005; Pfeifer et al. 2006; Reber et al. 2006; Wagner and Laufer 2006). This may be achieved by small molecular anti-cytokine agents inhibiting cytokine production, which target p38 mitogen activated protein (MAP) kinase, TNF- α converting enzyme (TACE), or IL-1 β converting enzyme (ICE).

Several so-called “biologicals” are in clinical use:

Etanercept (Enbrel), a fully human soluble TNF receptor fusion protein consisting of the extracellular ligand-binding domain of the 75-kDa receptor for human tumor necrosis factor- α and the constant portion of human IgG1 (Jarvis and Faulds 1999; Pugsley 2001; Scallon et al. 2002; Agnholt et al. 2003; Cole and Rabasseda 2004; Goffe 2004; Moe et al. 2004; Vallejo et al. 2005). The compound has been approved for treatment of psoriasis, psoriatic arthritis, ankylosing spondylitis and rheumatoid arthritis.

Infliximab (Remicade), a chimeric anti human TNF- α monoclonal antibody (Scallon et al. 2002; Agnholt et al. 2003; Di Sabatino et al. 2004; Wagner et al. 2004; Panaccione et al. 2005; Shen et al. 2005; Pfeifer et al. 2006). The compound is used for treatment of rheumatoid arthritis and Crohn’s disease.

Adalimumab (HUMIRA), a recombinant human anti-human TNF- α monoclonal antibody (Gordon et al. 2005; Aggarwal et al. 2006; Scheinfeld 2006; Shen et al. 2006). The compound is used for treatment of rheumatoid arthritis and psoriatic arthritis.

Imatinib mesylate (STI571, Gleevec), a kinase inhibitor of TNF- α production (Kilic et al. 2000; Traxler et al. 2001; Dietz et al. 2004; Kaelin 2004; Lassila et al. 2005; Wolf et al. 2005; Adcock et al. 2006). The compound has been found to be active in the treatment of chronic myelogenous leukemia, gastrointestinal stromal tumors, eosinophilic disorders, and systemic mast cell disease.

Omalizumab (Xolair), a recombinant humanized monoclonal antibody which specifically binds the C ϵ 3 domain of IgE, the site of high-affinity IgE receptor binding (Easthope and Jarvis 2001; Anonymous 2002; Johansson et al. 2002; Davis 2004; Richards et al. 2004; Belliveau 2005; D’Amato 2006). The compound is used for treatment of bronchial asthma and allergic rhinitis.

Anakinra (Kineret) is a specific recombinant human interleukin-1 receptor antagonist that differs from naturally occurring IL-1 receptor antagonist by the presence of a methionine group (Cvetkovic and Keating 2002; Fleischmann et al. 2004; Le and Abbenante 2005; Waugh and Perry 2005). The compound is effective in patients with active rheumatoid

arthritis, either when given alone or in combination with methotrexate.

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H.3.1.8.2

Inhibition of TNF- α Release

PURPOSE AND RATIONALE

There are two distinct types of tumor necrosis factors, TNF-alpha (cachectin) and TNF-beta (lymphotoxin), with biological activities going beyond the necrosis of tumor cells. Some of the known activities include the induction of interleukin 1, activation of PMNs, modulation of endothelial cell functions, and augmentation of specific immune functions. The complex sequence of hemodynamic and metabolic collapse, which leads to shock and death during lethal endotoxemia, appear to represent the response of the infected host to the acute, systemic release of TNF-alpha. Thus, drugs that antagonize the activity of this mediator could be of clinical value in combating its fatal effects.

PROCEDURE

Twenty hours before the initiation of the experiments, L 929 cells are harvested from stock cultures and are plated in 96 well culture plates (2×10^4 cells/well) and incubated at 37°C and 5% CO₂ in air. For each group 6 wells are set up. The cells are then preincubated for 30 min with test substances or solvent before TNF-alpha is added (between 1 and 10 IU/well). After an additional incubation time of 20 h, the culture plates are flicked out and the remaining living cells are lysed by the addition of bidistilled water (100 μ l). After 30 min incubation at room temperature, 100 μ l of LDH reagent are given to each culture well. After 15 min, the enzyme activity is determined photometrically at 490 nm.

EVALUATION

The percent inhibition is calculated according to the formula:

$$\% \text{ inhibition} = 100\% \times \frac{\text{ext. test group} - \text{ext. spontaneous lysis}}{\text{ext. positive control} - \text{ext. spontaneous lysis}}$$

The positive control is the group which receives vehicle and TNF-alpha. The spontaneous lysis is based on cultures which receive vehicle without TNF-alpha.

MODIFICATIONS OF THE METHOD

Maloff and Delmendo (1991) measured the binding of tumor necrosis factor (TNF- α) to the human TNF receptor. Membranes were prepared from HeLa S3 human cervical epithelioid carcinoma cells. An aliquot of 0.2 mg of membrane preparation was incubated with 62 pM [¹²⁵I]TNF- α for 3 h at 4°C. Nonspecific binding was measured in the presence of 50 nM TNF- α . Membranes were filtered and washed 3 times and the filters were counted to determine the bound [¹²⁵I]TNF- α .

Golebiowski et al. (2005) tested pyrazolone-based cytokine synthesis inhibitors for the inhibition of TNF- α production using lipopolysaccharide-stimulated monocytic cells. Duplicate cultures of human monocytic cells (2.0×10^6 /well) were incubated for 15 min in the presence or absence of various concentrations of inhibitor before the stimulation of cytokine release by the addition of lipopolysaccharide (1 μ g/ml). The amount of TNF- α released was measured 4 h later using an ELISA system.

Kumar et al. (1997) described homologs of CSBP/p38 MAP kinase, their activation, as well as substrate specificity and sensitivity to inhibition by pyridyl imidazoles.

McLay et al. (2001) reported the discovery of a p38 MAP kinase inhibitor displaying a good oral anti-arthritic efficacy.

Ignar et al. (2003) described the regulation of TNF- α secretion by a specific melanocortin-1 receptor peptide agonist.

Kinases Assay

The p38 enzyme assay is carried out at room temperature for 1 h, using 40 ng/well of the mouse enzyme. The substrate, 50 μ g/ml ATF-2 transformation factor, is coated onto 96-well plates, and the assay is carried out in 25 mM Hepes buffer, pH 7.7 containing 25 mM magnesium chloride, 2 mM dithiothreitol, 1 mM sodium orthovanadate and 100 μ M ATP. Phosphorylated ATF-2 is quantitated using a phospho-specific ATF-2 primary antibody (rabbit anti-human) followed by a europium-labeled secondary antibody (sheep anti-rabbit IgG) with addition of the DELFIA enhancement solution resulting in fluorescence. ERK was measured using a [³³P]ATP filtration assay format using for substrates myelin basic protein. ZAP-70, Syk and Lck kinase activities were measured using the homogeneous time-resolved fluorescence methodology (HTRF) with the catalytic domains of each of the tyrosine kinases, biotinylated, specific peptide substrates, streptavidin-linked APC and europium cryptate-conjugated anti-phosphotyrosine antibody.

Monocyte TNF- α Release Assay

Adherent human monocytes (100,000 cells/well) were incubated with LPS (10 ng/ml) in the absence and presence of compound for 18 h. Individual experiments were carried out in quadruplicate samples. TNF- α was measured by sandwich ELISA and IC₅₀ values calculated for the activity of individual compounds. IC₅₀ values shown from repeat experiments are means \pm SEM.

Mouse TNF- α Release Assay

Compound was administered orally to BALB/c mice 30 min prior to LPS (0.1 mg/kg i.p.) challenge. Serum TNF- α levels were determined 90 min after LPS insult. Results represent means \pm SEM.

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H.3.1.8.3

Effect of TNF- α Binding

PURPOSE AND RATIONALE

Several authors compared the effects of the prototypes of the tumor necrosis factor (TNF) antagonists infliximab or adalimumab and etanercept (Vuolteenaho et al. 2002; Kirchner et al. 2004; Shen et al. 2005).

Scallon et al. (2002) compared the binding and functional properties of the two prototypes of the TNF antagonists infliximab and etanercept-

Although both infliximab and etanercept are potent neutralizers of TNF bioactivity, there are fundamental differences in their molecular structures, their binding specificities, and the manner in which they neutralize TNF. Infliximab is a chimeric monoclonal antibody (mAb) with murine variable regions and human IgG1 and κ constant regions (Knight et al. 1993). The size (149 kDa) and structure of infliximab are therefore similar to those of naturally occurring antibodies. Etanercept is a fusion protein made up of the extracellular domain of the p75 TNF receptor (CD120b) and the hinge and Fc domains of human IgG1 (Mohler et al. 1993), a structure distinct from any known naturally occurring molecule. Importantly, infliximab is not known to bind to any antigen other than TNF, whereas etanercept binds equally well to both TNF and lymphotoxin α (LT α), consistent with observations reported for the cellular p75 TNF receptor (Schall et al. 1990; Smith et al. 1990). Each infliximab molecule is capable of binding to two TNF molecules, and up to three infliximab molecules can bind to each TNF ho-

motrimer, thereby blocking all receptor binding sites on TNF. In contrast, it is believed that the bivalent etanercept molecule forms a 1:1 complex with the TNF trimer in which two of the three receptor binding sites on TNF are occupied by etanercept, and the third receptor binding site is open. In addition, the p75 TNF receptor is known to have fast rates of association and dissociation with TNF (Evans et al. 1994), which suggests that etanercept may only transiently neutralize the activity of an individual TNF molecule.

PROCEDURE

Cell Culture

KYM-1D4 cells that endogenously express TNF receptors (Butler et al. 1994) were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FBS. Human umbilical vein endothelial (HUVE) cells from Cell Systems (Seattle, Wash., USA) were maintained in HUVE cell medium supplied by Cell Systems. K2 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% FBS, 2 mM L-glutamine, 0.5 µg/ml mycophenolic acid, 2.5 µg/ml hypoxanthine, and 50 µg/ml xanthine. All cells were cultured in a humidified incubator maintained at 37°C and 5% CO₂.

Binding to Monomer Subunits of TNF

Dimethylsulfoxide (DMSO) was added to ¹²⁵I-TNF (40–60 µCi/µg; 1.48–2.2 MBq/µg) to a final concentration of 10% DMSO and incubated at 20°C for 30 min to allow dissociation of TNF trimers. The mixture was passed over a 10 × 300-mm Superose 12 column equilibrated with PBS, and ¹²⁵I-TNF trimer and monomer were collected separately. Polystyrene 96-well microtiter plates were coated by incubating 50 µl of 1 µg/ml of infliximab, etanercept, or an isotype-matched, negative control antibody (cM-T412) in the wells overnight at 4°C. After washing with PBS-0.05% Tween 20 (PBS-T), all wells were blocked for 1 h at 37°C with PBS-1% bovine serum albumin and washed three times with PBS-T. Triplicate wells were then incubated with ¹²⁵I-TNF trimer [0.4 µCi (14.8 kBq), 10 ng/ml] or ¹²⁵I-TNF monomer [0.1 µCi (3.7 kBq), 2.5 ng/ml] alone or with 5 µg/ml unlabeled TNF. After 1 h at 37°C, the wells were washed with PBS-T and counted for ¹²⁵I.

Binding Assay to Measure Stability of Complexes with Soluble TNF

Each well of a 96-well enzyme immunoassay plate was incubated overnight at 4°C with 100 µl of 0.1 M carbonate, pH 9.6, containing 10 µg/ml goat anti-human

γ Fc antibody. Plates were washed three times with PBS-T and then incubated for 1 h at 37°C in blocking buffer (10 mM HEPES, pH 7.5, containing 0.1% porcine gelatin, 150 µl/well). Wells were incubated for 1 h at 37°C with 100 µl/well of blocking buffer containing 1 µg/ml infliximab or etanercept. Plates were washed three times with PBS-T, and then all TNF binding sites were saturated by incubating the wells for 1 h at 37°C in 100 µl/well of blocking buffer containing 10 ng/ml ¹²⁵I-TNF (40–60 µCi/µg). Wells were washed three times with PBS-T and then filled with 100 µl/well of blocking buffer alone or containing an excess of soluble, unlabeled competitor such as infliximab, etanercept, or human TNF, and subsequently incubated at 37°C. At the indicated time points, triplicate wells were washed three times with PBS-T to remove free ¹²⁵I-labeled TNF. The last wash was aspirated and replaced with 50 µl of scintillation fluid and the entire plate counted in a Packard TopCount gamma counter.

Assay for Bioactivity of Dissociated Soluble TNF

Microtiter plates were coated with goat anti-human γ Fc antibody and used to capture etanercept as described above. Wells were washed three times with PBS-T and incubated with 100 µl of 10 ng/ml unlabeled human TNF in 100 µl/well of blocking buffer for 1 h at 37°C. Wells were washed three times with KYM media, filled with 100 µl of KYM media, and 500 ng/ml mouse TNF was added to each well as a competitor. After a 1-h incubation at 37°C, the soluble fraction was removed and pre-incubated for 1 h in fresh wells with either no mAb, 10 µg/ml anti-human TNF mAb (infliximab), 85 µg/ml anti-mouse TNF mAb (cV1q huG3), or a combination of 10 µg/ml anti-human TNF and 85 µg/ml anti-mouse TNF mAb. After the pre-incubation, the soluble fractions were added to cultures of KYM-1D4 cells (50,000 cells/well in a 96-well plate) and the cells incubated for 16 h at 37°C in the presence of 0.5 µg/ml actinomycin D. To quantitate cell viability, MTT dye was added to a final concentration 0.5 mg/ml and the cells incubated at 37°C for 4 h. The medium was aspirated and 100 µl of 100% DMSO was added to the cells. The difference between the absorbance at 550 and 650 nm was then determined.

HUVE Cell Assay to Measure Stability of Complexes with Soluble TNF

Infliximab or etanercept was mixed with 1 µg/ml human TNF at 10:1 or 30:1 M ratios in HUVE cell medium and incubated for 30 min at 37°C. Serial di-

lutions of the preformed complexes were then added to confluent HUVE cells cultured in 96-well plates. Cells were incubated with the preformed complexes in 100 μ l of HUVE cell medium for 4 h at 37°C and then washed three times with HBSS. The cells were then incubated for 1 h at 37°C in HBSS containing 1 μ g/ml 125 I-labeled anti-E-selectin (20 μ Ci/ μ g). Cells were washed three times with HBSS, the last wash was aspirated and replaced with 30 μ l of scintillation fluid, and the entire plate was counted in a Packard TopCount gamma counter.

Binding Assay to Measure Stability of Complexes with Transmembrane TNF

K2 cells, which stably express an uncleavable and thus permanently transmembrane (tm) form of TNF, were seeded at a density of 5×10^4 cells/well in a 96-well round-bottom plate in 100 μ l of IMDM, 5% FBS. Subsequently, 125 I-labeled infliximab or 125 I-labeled etanercept (both at 8.5 μ Ci/ μ g) was added to a final concentration of 0.5 μ g/ml (enough to saturate all TNF binding sites on the cells). After a 1-h incubation at 25°C, unbound infliximab and etanercept were removed by washing three times with IMDM medium. Fresh IMDM, 5% FBS medium (100 μ l) alone, or containing 50 μ g/ml of an unlabeled soluble competitor, was added to the cells. Soluble competitors were either infliximab or etanercept for samples treated with radiolabeled infliximab and one of infliximab, etanercept, or human LT α for samples treated with radiolabeled etanercept. The cells were then incubated at 37°C in 5% CO₂. At different time points, cells in selected wells were washed with PBS, and the number of counts bound to the cells determined using a gamma counter (PerkinElmer Wallac, Wellesley, Mass., USA).

Characterization of Infliximab and Etanercept Binding to tmTNF

K2 cells or TNF-negative Sp2/0 control cells were seeded in 96-well round-bottom plates at a density of 5×10^4 cells/well in IMDM, 5% FBS. Varying amounts of 125 I-labeled infliximab (23.4 μ Ci/ μ g) or 125 I-labeled etanercept (22.4 μ Ci/ μ g) were added to the cells. After a 16-h incubation at 4°C, cells were washed four times with culture medium (IMDM, 5% FBS), the last wash was aspirated, and 50 μ l of culture medium was added to each well. The cells were then removed with cotton swabs and the number of counts per well was determined using a gamma counter (PerkinElmer Wallac). The resulting binding data were analyzed by non-linear regression using Prism software (GraphPad Software, San Diego, Calif., USA).

HUVE Cell Assay to Compare Ability to Inhibit tmTNF Bioactivity

K2 cells or Sp2/0 control cells were seeded in 96-well round-bottom plates at a density of 1×10^5 cells/well in IMDM, 5% FBS. Varying amounts of infliximab or etanercept in IMDM, 5% FBS were added and the mixture incubated for 1 h at 37°C. This mixture was then added to confluent cultures of HUVE cells in 96-well plates. The resulting cell-cell mixture was incubated for an additional 4 h at 37°C in a 5% CO₂ incubator. Cells were then washed three times with HBSS and incubated for 1 h with 1 μ g/ml 125 I-anti-E-selectin (20 μ Ci/ μ g). Cells were washed three times with HBSS, the last wash was aspirated and replaced with 30 μ l of scintillation fluid, and the plate counted in a Packard TopCount gamma counter.

EVALUATION

Data were analyzed using a paired Student's *t*-test to determine whether there was a statistically significant difference between the capacities of infliximab and etanercept to block the bioactivity of tmTNF.

MODIFICATIONS OF THE METHOD

Maloff and Delmendo (1991) developed high-throughput radioligand binding assays for interleukin 1- α (IL-1- α) and tumor necrosis factor (TNF- α) in isolated membrane preparations.

Zhang et al. (2002) described identification and characterization of a dual tumor necrosis factor converting enzyme/matrix metalloprotease inhibitor for the treatment of rheumatoid arthritis.

Transgenic mice expressing human tumor necrosis factor develop severe polyarthritis (Keffer et al. 1991; Kollias et al. 1999; Kontoyiannis et al. 1999; Mijatovic et al. 2000; Akassoglou et al. 2003; Li and Schwarz 2003). A targeting vector containing a genomic fragment encoding the entire TNF- α with the ARE-containing 3' UTR was replaced with the 3' UTR from the β -globin gene. This mutation increases the stability and translational efficiency of TNF- α mRNA and thus results in chronic TNF- α over-expression that leads to severe erosive polyarthritis. Administration of anti-TNF- α antibodies completely prevents the disease.

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H.3.1.9

Binding to Interferon Receptors

PURPOSE AND RATIONALE

The interferons (IFNs) were discovered in 1957 as biological agents interfering with virus replication. They are a family of secreted proteins occurring in vertebrates and can be classified as cytokines. The IFNs are multifunctional and are components of the host defense against viral and parasitic infections and certain tumors. They affect the functioning of the immune system in various ways and also affect cell proliferation and differentiation.

IFNs were initially classified by their sources as leukocyte, fibroblast, and immune IFNs. Leukocyte and fibroblast IFNs, together, were also designated as Type 1 IFNs and immune IFN as Type 2 IFN. The recent nomenclature designates leukocyte IFNs as IFN- α and IFN- ω (earlier α -1 and α -2, respectively) fibroblast IFN as IFN- β , and immune IFN as IFN- γ .

Interferons bind to receptors on the cell surface and induce the synthesis of specific proteins. Littman et al. (1985) found that recombinant IFN- γ produced in bacteria, which is not glycosylated, binds to cellular receptors with an affinity similar to that of natural IFN- γ .

PROCEDURE

Human lymphoblastoid cells (Daudi, MOLT-4 and Raji) are grown in stationary cultures in Dulbecco's medium with 10% heat-inactivated horse serum. HeLa cells are grown in Eagle's medium with 7% horse serum.

The following interferons are used: Purified recombinant interferon- γ (rIFN- γ) (Genentech, antiviral activity 1.2×10^7 units/mg); natural human INF- β (Interferon Working Group of the NCI, antiviral activity 2×10^5 roentgen units/mg); rIFN-2 α (Schering, antiviral activity 2×10^8 reference units/mg); rIFN- β (Cetus Corp., antiviral activity 2.6×10^8 reference units/mg).

Fifty micrograms of rIFN- γ are reacted for 2 h at 0°C with 1 mCi of ^{125}I -Bolton-Hunter reagent

(2000 Ci/mmol) in 0.25 ml of sodium borate buffer, pH 8.0. The reaction is stopped by the addition of glycine to a final concentration of 0.2 M and applied to a 26 × 0.7 cm column of Sephadex G-75 equilibrated with phosphate-buffered saline, pH 7.4, containing 0.25% gelatin. The reaction vial is washed with 20- μ l aliquots of this buffer containing 40% ethylene glycol and then with buffer alone. The washes are added to the column and 0.32 ml fractions are collected. The fractions containing 125 I-rIFN- γ are pooled and diluted with 1/10 volume of 10-fold concentrated Eagle's medium containing 10 mg/ml bovine serum albumin and 0.1 mM dithiothreitol.

Cells harvested from exponentially growing cultures are centrifuged and resuspended at 8×10^6 /ml in their own medium supplemented with 10 mM HEPES buffer, pH 7.4. Standard binding assays contain 3 to 5×10^6 cells and 0.46 nM 125 I-rIFN- γ . At the end of the reaction, the cells are centrifuged through 10% sucrose at 10,000 rpm in Microfuge tubes and the cell pellet is counted. A blank value is determined by incubating and processing in the same way an equal amount of 125 I-rIFN- γ in the absence of cells; this blank is subtracted from the cpm bound.

EVALUATION

The binding data are analyzed using the LIGAND program developed by Munson and Rodbard (1980).

MODIFICATIONS OF THE METHOD

Blatt et al. (1996) described the biological activity of consensus interferon, a wholly synthetic type I interferon, developed by scanning several interferon-alpha nonallelic subtypes and assigning the most frequently observed amino acid in each position.

IFN- τ , a new class of type I interferon was described by Pontzer et al. (1994), Alexenko et al. (1997, 1999), Martal et al. (1998), Swann et al. (1999).

Thiam et al. (1998) reported the agonist activities of a lipopeptide derived from INF- γ on murine and human cells by analysis and quantification of cell surface markers using flow cytometry and cell-ELISA.

Bosio et al. (1999) reported efficacy of type I interferon in cytomegalovirus infections *in vivo*. Oral administration of type I interferons (murine INF- α and INF- β) reduced early replication of murine cytomegalovirus in both the spleen and liver of infected BLB/c mice.

Tovey and Maury (1999) found a marked antiviral activity of murine interferon- α/β or individual recombinant species of murine INF- α , INF- β , or INF- γ

or recombinant human INF- α 1–8 in mice challenged systemically with a lethal dose of encephalomyocarditis virus, vesicular stomatitis virus, or varicella zoster virus. Oromucosal administration of INF- α also exerted a marked antitumor activity in mice injected i.v. with highly malignant Friend erythroleukemia cells or other transplantable tumors, such as L1210 leukemia, the EL4 tumor, or the highly metastatic B16 melanoma.

To gain more insight into similarities of different INF- α species, Viscomi et al. (1999) evaluated neutralization and immunoactivity of a variety of INF preparations with various monoclonal antibodies obtained through immunization with recombinant, lymphoblastoid, and leukocyte INF- α .

Reporter transgenic mice expressing the luciferase gene under the control of separate TCR-response elements from the INF- γ promoter or expressing the green fluorescent protein gene under the control of an INF- γ minigene were employed by Zhang et al. (1999) to explore the basis for IL-12 regulation of INF- γ gene transcription.

Poynter and Daynes (1999) studied the influence of constitutively expressed INF- γ on age-associated alterations in inducible nitric oxide synthase regulation using cell cultures from mouse spleen for nitrite and cytokine analysis.

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H.3.1.10

Chemokine Antagonism

PURPOSE AND RATIONALE

The human chemokine system comprises about 50 distinct chemokines and 20 G-protein-coupled chemokine receptors (Rossi and Zlotnik 2000; Salusto et al. 2000; Zlotnik and Yoshie 2000; Fernandez and Lolis 2002; D'Ambrosio et al. 2003; Houshamand and Zlotnik 2003; Ono et al. 2003; Proudfoot et al. 2003; Chen et al. 2004; Haringman and Tak 2004; Cunha et al. 2005). The biological activities of chemokines range from the control of leukocyte trafficking in basal and inflammatory conditions to regulation of hematopoiesis, angiogenesis, tissue architecture and organogenesis. Grainger and Reckless (2005) studied the anti-inflammatory effects of broad-spectrum chemokine inhibitors. Several groups described CC chemokine receptor-1 antagonists (Liang et al. 2000a, 2000b; Naya et al. 2001; Eltayeb et al. 2003; Gladue et al. 2006). CC chemokine receptor-3 (CCR3) antagonists were reported by De Lucca et al. (2005) and Fryer et al. (2006). CC chemokine receptor-5 (CCR5) antagonists were described by Rosi et al. (2005) and Saita et al. (2005).

De Lucca et al. (2005) described the discovery of CC chemokine receptor-3 (CCR3) antagonists with picomolar potency.

PROCEDURE

Biological Assays

CCR3-Receptor Binding

Millipore filter plates (no. MABVN1250) are treated with 5 μ g/ml protamine in phosphate-buffered saline, pH 7.2, for 10 min at room temperature. Plates are washed three times with phosphate-buffered saline and incubated with phosphate-buffered saline for 30 min at room temperature. For binding, 50 μ l of binding buffer (0.5% bovine serum albumen, 20 mM HEPES buffer, and 5 mM magnesium chloride in RPMI 1640 media) with or without a test concentration of a compound present at a known concentration is combined with 50 μ l of 125 I-labeled human eotaxin (to give a final concentration of 150 pM radioligand) and 50 μ l of cell suspension in binding buffer containing 5×10^5 total cells. Cells used for the binding assay are CHO cell lines transfected with a gene expressing human CCR3 (Daugherty et al. 1996). The mixture of compound, cells, and radioligand is incubated at room temperature for 30 min. Plates are placed onto a vacuum manifold, vacuum is applied, and the plates are washed three times with binding buffer with 0.5 M NaCl added. The plastic skirt is removed from the plate, and the plate is allowed to air-dry; the wells are punched out, and the radioactivity counted (cpm).

EVALUATION

The percent inhibition of binding is calculated using the total count obtained in the absence of any competing compound or chemokine ligand and the background binding determined by addition of 100 nM eotaxin in place of the test compound.

Human Eosinophil Chemotaxis Assay

Neuroprobe MBA96 96-well chemotaxis chambers with Neuroprobe poly(vinylpyrrolidone)-free polycarbonate PFD5 5- μ m filters in place are warmed in a 37°C incubator prior to the assay. Freshly isolated human eosinophils are suspended in RPMI 1640 with 0.1% bovine serum albumin at 1×10^6 cells/ml and warmed in a 37°C incubator prior to the assay. A 20 nM solution of human eotaxin in RPMI 1640 with 0.1% bovine serum albumin is warmed in a 37°C incubator prior to the assay. The eosinophil suspension and the 20 nM eotaxin solution are each mixed 1:1 with prewarmed RPMI 1640 with 0.1% bovine serum albumin with or without a dilution of a test compound that is at twofold the desired final concentration. The filter is separated, and the eotaxin/compound mixture is

placed into the bottom part of the chemotaxis chamber. The filter and upper chamber are assembled, and 200 μ L of the cell suspension/compound mixture is added to the appropriate wells of the upper chamber. The upper chamber is covered with a plate sealer, and the assembled unit is placed in a 37°C incubator for 45 min. After incubation, the plate sealer is removed and all remaining cell suspension is aspirated off. The chamber is disassembled, and unmigrated cells are washed away with phosphate-buffered saline and then the filter is wiped with a rubber-tipped squeegee. The filter is allowed to completely dry and stained with Wright Giemsa. Migrated cells are enumerated by microscopy.

Calcium Mobilization Assay

Intracellular calcium flux was measured as the increase in fluorescence emitted by the calcium-binding fluorophore, fluo-3, when preloaded cells were stimulated with CCR3 ligand. Freshly isolated eosinophils were loaded with fluorophore by resuspending them in a HEPES-buffered PBS solution containing 5 μ M fluo-3 and incubating for 60 min at 37°C. After being washed twice to remove excess fluorophore, cells were resuspended in binding buffer (without phenol red) and plated into 96-well plates at 2×10^5 /well. Plates were placed individually in a FLIPR-1 (Molecular Devices) that uses an argon-ion laser to excite the cells and robotically adds reagents while monitoring changes in fluorescence in all wells simultaneously. To determine the IC₅₀, compound or buffer alone was added and cells were incubated for 5 min; eotaxin was then added to a final concentration of 10 nM. The fluorescence shift was monitored, and the base-to-peak excursion was computed automatically. All conditions were tested in duplicate, and the mean shift per condition was determined. The inhibition achieved by graded concentrations of compound was calculated as a percentage of the compound-free eotaxin control.

MODIFICATIONS OF THE METHOD

Chen et al. (1998) reported *in vivo* inhibition of CC and CX₃C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar–Kyoto (WKY) rats by the viral protein vMIP-II.

Ruth et al. (2001) investigated fractalkine, a chemokine, in rheumatoid arthritis and in rat adjuvant-induced arthritis. Fractalkine in vascular biology was discussed by Umehara et al. (2004).

Laudanna and Constanin (2003) described new models of intravital microscopy for analysis of che-

mokine receptor-mediated leukocyte vascular recognition.

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H.3.1.11

Influence of Peroxisome Proliferator-Activated Receptors (PPARs) on Inflammation

PURPOSE AND RATIONALE

Peroxisome proliferator-activated receptors (PPARs) play an important role not only in lipid metabolism and diabetes but also in the inflammation process (Devchand et al. 1996; Delerive et al. 2001; Cabrero et al. 2002; Clark 2002; Blanquart et al. 2003; Moller and Berger 2003; Nencioni et al. 2003; Tai et al. 2003; Woerly et al. 2003; Diep et al. 2004).

Jiang et al. (1998) reported that PPAR- γ antagonists inhibit production of monocyte inflammatory cytokines.

PROCEDURE

Monocyte Preparation

Human monocytes are isolated from freshly collected buffy-coat preparations of whole human blood. The mononuclear cell fraction is prepared by dilution with an equal volume of phosphate-buffered saline (PBS) at room temperature and layered over a solution containing 3 ml Ficoll-Hypaque per 10 ml blood and PBS and centrifuged for 10 min at 900 g. The mononuclear cell layer is transferred to a fresh tube, mixed with 3 vols of PBS, and centrifuged for 10 min at 400 g. The supernatant is removed and the dilution and centrifugation are repeated three times. Mononuclear cells are resuspended in RPMI medium 1640, counted and diluted to 5×10^6 cell per ml, after which 1 ml is transferred to each well of a 24-well tissue culture plate and incubated for 37°C in a 5% CO₂ humidified incubator. The non-adherent cells are removed and the monocytes washed once with PBS before adding 1 ml of fresh RPMI medium 1640 with 10% fetal bovine serum. Experiments are initiated on the day blood is collected, and all manipulations are carried out under endotoxin-free conditions.

Cytokine Assay

Monocytes in fresh medium are treated with inducers and candidate induction inhibitors at the time of culture initiation. Medium is collected from triplicate wells 18–20 h after the test compounds are added. Supernatant concentrations of TNF- α , IL-6 and IL-1 β are measured by ELISA.

RNA Blot

Total RNA of human monocyte cultures is isolated 15 h after stimulation with 25 nM PMA in the absence or presence of PPAR γ agonists. RNA blot analyses are performed with standard procedures and labeled probes prepared from human TNF- α and GAPDH cDNA with the Megaprime DNA labeling Kit (Amersham Life Science).

Luciferase Assay

U937 cells (2×10^7) are transfected with 10–20 μ g luciferase reporter DNA by electrophoresis at 875 V cm⁻¹, 960 μ F (Bio-Rad Laboratories). Transfected cells are allowed to recover for 1 h and triplicate samples are either untreated or treated with 25 nM PMA in the absence or presence of indicated drugs. Luciferase activity is measured 18–36 h later using the Dual-luciferase reporter assay system (Promega) with the pRL-TK vector as an internal reporter control.

EVALUATION

To obtain IC_{50} values, data are fitted to a four-parameter exponential and the derived parameters used to calculate the concentration at which 50% of the maximal activity is observed.

MODIFICATIONS OF THE METHOD

Kojo et al. (2003) evaluated human PPAR subtype selectivity of a variety of anti-inflammatory drugs based on a novel assay for PPAR δ (β).

PROCEDURE

Plasmids

The cDNAs for human PPAR α , δ , and γ were synthesized using the polymerase chain reaction (PCR) with human liver cDNA for human PPAR α , human heart cDNA for human PPAR δ (β), and human fat cell cDNA for human PPAR γ 1 as templates. Retinoid X receptor (RXR) α expression plasmids were constructed by inserting a coding sequence of RXR α into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, Calif., USA). The cDNA for human RXR α was synthesized with human kidney cDNA as a template. The template cDNAs used were purchased from Clontech Laboratories (Palo Alto, Calif., USA). The amplified cDNA fragments were cloned into pCRII (Invitrogen) and the nucleotide sequences of the cDNAs were determined by the dideoxy chain termination method using an automated laser fluorescent DNA sequencer. Multiple clones of each cDNA were sequenced and artifacts generated by the *Taq* polymerase used in the PCR were revised by replacement of the specified region among the clones. The PPAR and RXR α expression plasmids were constructed by inserting each full-length cDNA at a multiple cloning site of the mammalian expression vector pCDM8, pcDNA1, or pcDNA3.1 (+) (Invitrogen). Coactivator expression plasmids pcDNA3.1-CBP and pcDNA3.1-SRC-1 were provided by Fujimura and Aramori of the Pharmacological Research Laboratories of Fujisawa Pharm. The human RXR γ expression plasmid was purchased from Invitrogen.

The reporter gene plasmid pGVPPRELuc-1 was constructed as follows. Three copies of a 33-bp PPRE identical to that of acyl CoA oxidase were first cloned at the *SalI* site of pBLCAT-2 to generate pBLPPRECAT-1. The luciferase reporter plasmid pGVPPRELuc was constructed by inserting the PPRE-containing fragment of pBLPPRECAT-1 at a multiple cloning site of PGV-P2 (Wako Pure Chemical, Osaka).

Gal4-PPAR δ (β) fusion expression plasmid for one-hybrid assay was constructed as follows: full-

length PPAR δ (β) cDNA and PPAR δ (β)-LBD cDNA were prepared by PCR amplification using pCDM8-hPPAR δ as a template, and the amplified cDNA was cloned into a multiple cloning site of the pBIND vector (Promega, Madison, Wis., USA). The nucleotide sequence of each fusion expression plasmid was checked with an automated DNA sequencer. Reporter plasmid pG5luc for the one-hybrid assay was purchased from Promega.

Transient Transfection Assay

The African green monkey fibroblast cell line CV-1 was obtained from ATCC (Manassas, Va., USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, Md., USA) supplemented with penicillin, streptomycin, and 10% heat-inactivated fetal calf serum. Cells were seeded at 2×10^5 per well of 6-well culture dishes and after overnight culture transiently transfected with 1 μ g each of pGVPPRELuc luciferase reporter plasmid, PPAR expression plasmid, and RXR α expression plasmid together with the control *Renilla* luciferase expression plasmid RL-TK (Promega) using Lipofectamine 2000 (Gibco-BRL). Coactivator expression plasmid was also included when a transfection was performed for the PPAR δ assay. For the one-hybrid assay, cells were transfected with 1 μ g each of pG5luc reporter plasmid and Gal4-PPAR δ fusion expression plasmid with RL-TK control plasmid. Cells were harvested 4 h after transfection and plated again at 1.6×10^4 per well onto 96-well plates. The drugs dissolved in dimethyl sulfoxide were added to the culture and the cells were incubated at 37°C for 24 h. After being washed with PBS(-), cells were lysed with PLB (passive lysis buffer) (Promega) and the lysates were used for reporter assays. Expression of the reporter was measured by the activity of firefly luciferase using the dual luciferase reporter assay system (Promega) and ARVO HTS 1420 multilabel counter (Amersham Biosciences) as a luminometer. Firefly luciferase activity was corrected for transfection efficiency based on the activity of internal control *Renilla* luciferase.

Bishop-Bailey and Warner (2003) found that PPAR γ ligands induce prostaglandin production in vascular smooth muscle cells and concluded that indomethacin acts as a peroxisome proliferator-activated receptor- γ antagonist.

Fahmi et al. (2001) reported that peroxisome proliferator-activated receptor γ activators, such as 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$, inhibit interleukin-1 β -induced nitric oxide and metalloproteinase 13 production in human chondrocytes.

Cheng et al. (2004) found that peroxisome proliferator-activated receptor γ , which is activated by ligands such as troglitazone or 15-deoxy- $\Delta^{12,14}$ -PGJ₂, inhibits interleukin-1 β -induced membrane-associated prostaglandin E₂ synthase-1 expression in human synovial fibroblasts by interfering with the early growth response protein Egr-1.

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H.3.1.12

Binding to Histamine H₄ Receptor

PURPOSE AND RATIONALE

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H₁ receptor couples mainly to G_{q/11}, thereby stimulating phospholipase C, whereas the H₂ receptor interacts with G_s to activate adenylyl cyclase. The histamine H₃ and H₄ receptors couple to G_i proteins to inhibit adenylyl cyclase, and to stimulate MAPK (Hough 2001).

The H₄ receptor is highly expressed in peripheral leukocytes and intestinal tissue, making this receptor an interesting target in inflammatory diseases (Fung-Leung et al. 2004; De Esch et al. 2005; Lim et al. 2006; Zhang et al. 2006). The new receptor was cloned and characterized by Oda et al. (2000). Liu et al. (2001b) reported comparison of histamine H₄ receptors in several species. Gbahou et al. (2006) compared the pharmacology of human histamine H₃ and H₄ receptors and described structure–activity relationships of histamine derivatives. Thurmond et al. (2004) described a potent and selective histamine H₄ antagonist with anti-inflammatory properties. Lim et al. (2005) evaluated histamine H₁-, H₂-, and H₃-receptor ligands at the human H₄ receptor and identified 4-methylhistamine as the first potent and selective H₄ receptor agonist.

PROCEDURE

Cell Culture

SK-N-MC cell lines, which stably express either the human H₃R (SK-N-MC/hH₃) or H₄R (SK-N-MC/hH₄) as well as a cAMP-responsive-element-(CRE-) driven β -galactosidase reporter gene SK-N-MC/hH₃ or SK-N-MC/hH₄ cells (Lovenberg et al. 1999; Liu et al. 2001a), were cultured in Eagle's minimum essential medium supplemented with 5% fetal calf serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 600 μ g/ml G418 at 37°C in 5% CO₂ and 95% humidity.

Radioligand Binding Assays

The SK-N-MC/hH₃ cell homogenates were incubated for 40 min at 25°C with approximately 1 nM [³H]-N-methylhistamine in 25 mM KPO₄ buffer and 140 mM NaCl (pH 7.4 at 25°C), with or without competing ligands, whereas the SK-N-MC/hH₄ cell homogenates were incubated 1 h at 37°C in 10 nM [³H]histamine and 50 mM Tris-HCl (pH 7.4 at 37°C), with or without competing ligands. Bound radioligands were col-

lected on 0.3% polyethyleneimine-pretreated Whatman GF/C, and washed three times with 3 ml of ice-cold washing buffer (4°C) containing 25 mM Tris-HCl and 140 mM NaCl (pH 7.4 at 4°C) for the hH₃R and 50 mM Tris-HCl (pH 7.4 at 4°C) for the hH₄R.

EVALUATION

Binding analysis of 10 nM [³H]JNJ 7777120 (test compound) and 0.1 nM [¹²⁵I]iodophenpropit to the hH₄R was performed with the same conditions as described for [³H]histamine. In saturation binding analysis, the non-specific binding of [³H]histamine or [³H]JNJ 7777120 was determined with 1 μM clobenpropit. The binding analysis of [³H]mepyramine and [¹²⁵I]iodoaminopotentidine binding to human H₁R and human H₂R, respectively, was performed according to Bakker et al. (2004). The binding data were analyzed with Prism 4.0 (GraphPad Software, San Diego, Calif., USA), and data are presented as mean ± SEM. Mouse and rat H₄R radioligand binding assays were performed according to Liu et al. (2001b).

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H.3.2

In Vivo Methods for Anti-inflammatory Activity

H.3.2.1

General considerations

The inflammatory process involves a series of events that can be elicited by numerous stimuli, e. g., infectious agents, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury. The response is accompanied by the clinical signs of erythema, edema, hyperalgesia and pain. Inflammatory responses occur in three distinct phases, each apparently mediated by different mechanisms:

- an acute, transient phase, characterized by local vasodilatation and increased capillary permeability,
- a subacute phase, characterized by infiltration of leukocytes and phagocytic cells,
- and a chronic proliferative phase, in which tissue degeneration and fibrosis occur.

According to these phases, pharmacological methods have been developed.

Methods for testing acute and subacute inflammation are:

- UV-erythema in guinea pigs
- Vascular permeability
- Oxazolone-induced ear edema in mice
- Croton-oil ear edema in rats and mice
- Paw edema in rats (various modifications and various irritants)
- Pleurisy tests
- Granuloma pouch technique (various modifications and various irritants)

The proliferative phase is measured by methods for testing granuloma formation, such as:

- Cotton wool granuloma
- Glass rod granuloma
- PVC sponge granuloma.

Furthermore, methods for testing immunological factors have been developed, such as:

- Adjuvant arthritis in rats (various modifications)
- Experimental allergic encephalomyelitis
- Schultz-Dale-reaction
- Passive cutaneous anaphylaxis
- Arthus type immediate hypersensitivity
- Delayed type hypersensitivity

(see Chapter I).

H.3.2.2

Methods for Testing Acute and Subacute Inflammation

H.3.2.2.1

Ultraviolet Erythema in Guinea Pigs

PURPOSE AND RATIONALE

The test was first described by Wilhelmi (1949) who was able to delay the development of ultraviolet erythema on albino guinea pig skin by systemic pretreatment with clinically equivalent doses of phenylbutazone and other nonsteroidal anti-inflammatory agents. The test procedure was further developed by Winder et al. (1958) and since that time modified by various investigators.

PROCEDURE

Albino guinea pigs (Pirbright white strain) of both sexes with an average weight of 350 g are used. Eighteen h prior testing, the animals are shaved on both flanks and on the back. Then they are chemically depilated by a commercial depilation product or by a suspension of barium sulfide. Twenty minutes later, the depilation paste and the fur are rinsed off in running warm water. On the next day, the test compound is dissolved (or suspended) in the vehicle and half the dose of the test compound is administered by gavage (at 10 ml/kg) 30 min before ultraviolet exposure. Control animals are treated with the vehicle alone. Four animals are used for each treatment group and control. The guinea pigs are placed in a leather cuff with a hole of 1.5 × 2.5 cm size punched in it, allowing the ultraviolet radiation to reach only this area. An original Hanau ultraviolet burner Q 600 is warmed up for about 30 min prior to use and placed at a constant dis-

tance (20 cm) above the animal. Following a 2 min ultraviolet exposure, the remaining half of the test compound is administered. The investigator has to protect himself/herself by gloves and ultraviolet glasses. The erythema is scored 2 and 4 h after exposure.

EVALUATION

The degree of erythema is evaluated visually by 2 different investigators in a double-blinded manner. The followings scores are given:

- 0 = no erythema,
- 1 = weak erythema,
- 2 = strong erythema,
- 4 = very strong erythema.

Animals with a score of 0 or 1 are considered to be protected. The scoring after 2 and after 4 h gives some indication of the duration of the effect. ED_{50} values can be calculated. Doses of 1.5 mg/kg indomethacin p.o., 4 mg/kg phenylbutazone p.o. and 60 mg/kg acetylsalicylic acid p.o. have been found to be effective.

CRITICAL ASSESSMENT

The test has the advantage of simplicity but needs training of the investigators. Attempts to use reflection photometers in order to eliminate subjective scoring were unsuccessful. Corticosteroids after systemic application are rather ineffective in this test, however, can be evaluated after topical administration. The test is not particularly useful to study the duration of the anti-inflammatory effect.

MODIFICATIONS OF THE TEST

Yawalkar et al. (1991) tested several steroids after local application in the ultraviolet-induced dermatitis inhibition in guinea pigs. Clobetasol propionate was more effective than hydrocortisone, halobetasol propionate was superior to both corticosteroids.

Woodward and Owen (1979) used the albino guinea-pig ear as the site of inflammation produced by UV radiation. Ear temperature, water content of the ear and vascular permeability were measured. Indomethacin, phenylbutazone and aspirin given subcutaneously were active but paracetamol was not.

Warren et al. (1993) studied the role of nitric oxide synthase and cyclo-oxygenase in the skin blood flow to UVB irradiation in the shaved dorsal skin of anesthetized male **Sprague Dawley rats** with a laser Doppler flow probe. Topical application of clobetasol-

17-propionate immediately after irradiation inhibited the 18 h UVB response in a dose-dependent manner.

Glohuber (1976) measured skin thickness using calipers in **hairless mice** after UV-irradiation of the back and treatment with anti-inflammatory drugs.

Woodbury et al. (1994), Kligman (1994) described a rapid assay of the anti-inflammatory activity of topical corticosteroids by inhibition of a UVA-induced neutrophil infiltration in hairless mouse skin. Skh-hairless mice were irradiated with UVA light on an area of 2×2 cm square on the dorsal trunk fore 200 min in anesthesia. Steroid treatment was once daily for 7 days. Irradiation was on the 8th day. Neutrophils were counted microscopically in punch biopsies.

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is induced by a phlogistic substance (Miles and Miles 1992). Mediators of inflammation, such as histamine, prostaglandins and leucotrienes are released following stimulation e. g. of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a consequence, fluid and plasma proteins are extravasated and edemas are formed. These effects are counteracted by H₁-antihistaminics, inhibitors of arachidonic acid metabolism and by leucotriene receptor antagonists. In addition, membrane-stabilizing drugs are able to reduce capillary permeability. Vascular permeability is increased by intracutaneous injection of the mast cell-degranulating compound 48/80. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evan's blue.

PROCEDURE

Male Sprague-Dawley rats with a body weight between 160 and 200 g are used. The ventral sides of the animal are shaved. Five ml/kg of a 1% solution of Evan's blue are injected intravenously. One hour later the animals are dosed with the test compound orally or intraperitoneally or with the vehicle. Ten animals are used for each test group and the control. Thirty minutes later, the animals are briefly anaesthetized with ether and 0.05 ml of a 0.01% solution of compound 48/80 are injected intracutaneously at 3 sites both at the left and ventral side. Ninety minutes after the injection of compound 48/80 the animals are sacrificed by ether anesthesia. The abdominal skin is removed and the dye-infiltrated areas of the skin are measured.

EVALUATION

The diameter of the dye-infiltrated areas is measured in millimeters in two perpendicular directions and the mean values of all injection sites in one animal are calculated. The percent inhibition in the treated animals as compared to the control group is calculated. A treated animal which shows values less than 50% of controls can be considered as positive. ED₅₀ values can be calculated in this way. Phenylephrine at a dose of 15 mg/kg has been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The test for vascular permeability is useful for characterization of a new anti-inflammatory compound.

Since compounds with sympathomimetic activity have a pronounced effect this test cannot be regarded as a primary screening test for anti-inflammatory products. Together with an observation of writhing or

H.3.2.2.2

Vascular Permeability

PURPOSE AND RATIONALE

The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which

“squirming” of mice, Whittle (1964) has proposed to be able to distinguish between narcotic and non narcotic analgesics.

MODIFICATIONS OF THE METHOD

Shionoya and Ohtake (1975) described a simple method for extraction of extravasated dye (Evan's blue) in the skin.

Frimmer and Müller (1962) presented a critical survey on the use of dye methods for quantitative determination of increased capillary permeability following intracutaneous injection of active substances.

McClure et al. (1992) used the Olympus CUE-2 Image Analyzer to quantify vascular permeability in the Miles assay in guinea pigs.

Zentel and Töpert (1994) used oxazolone-induced Evans blue extravasation for preclinical evaluation of topical corticosteroids. Female **NMRI-mice** were sensitized by topical application of 50 µl of 40% oxazolone in ethanol to 4 cm² of the left flank. After 13 days the animals were injected intravenously with 0.2 ml of 0.5% Evan's blue in water and 20 µl of 4% oxazolone in ethanol were topically applied to 6 cm² of the right flank immediately after injection. Three h later the challenged skin was treated with various corticosteroids in ointment. The animals were sacrificed 24 h after treatment and the challenged skin removed. Evans blue extravasation was measured spectrophotometrically at 623 nm.

Teixeira et al. (1993) studied acute inflammatory reactions in **guinea pig skin** measuring infiltration of ¹¹¹In-labelled eosinophils and neutrophils and edema formation by extravasation of ¹²⁵I-human serum albumin.

Fujii et al. (1996) quantified vascular permeability by the extravasation of pontamine sky blue in the skin of male ddY mice after subcutaneous injection of lipopolysaccharides.

Blackham and Woods (1986) measured extravasation of pontamine sky blue in the **mouse** peritoneal cavity.

Cambridge et al. (1996) investigated 6-hydroxy-dopamine-induced plasma extravasation in **rat skin** after intravenous injection of ¹²⁵I-human serum albumin and Evan's blue.

Rouleau et al. (1997) measured the inhibition of capsaicin-induced plasma extravasation by a histamine H₃ receptor agonist prodrug by analysis of extravasated Evan's blue in skin, eye conjunctiva, nasal mucosa, trachea, main bronchi, esophagus and urinary bladder of rats.

Watanabe et al. (1984) used fluorescein isothiocyanate-labeled bovine serum albumin as tracer to measure vascular permeability in the **carrageenin air pouch of rats**.

Collins et al. (1993) studied the pro-inflammatory properties of the human recombinant vascular permeability factor containing 165 amino acids in **rabbits**.

Urinary bladder cystitis induced by cyclophosphamide was used as model of intestinal inflammation and pain by several authors (Ahluwalia et al. 1994; Bon et al. 1996; Boucher et al. 1997; Alfieri and Gardner 1997). Male Wistar rats weighing 300–400 g were treated first with test drug or saline subcutaneously or intraperitoneally and then injected 5 min later with 150 mg/kg i.p. cyclophosphamide. One h later, anaesthesia was induced by 40 mg/kg i.p. pentobarbitone and 50 mg/kg Evans blue were injected into the jugular vein. Fifteen min later, the rat was exsanguinated by infusion of 50 ml saline into the left cardiac ventricle. The urinary bladder, the left kidney, the superior lobe of the left lung and approximately 1-cm portions of the duodenum and jejunum were removed and blotted before dry weighing. The content of Evans blue dye was determined by spectrophotometry at 620 nm after extraction in known volumes of formamide at 60°C for 60 h.

Ferrets were treated in the same way, but the dose of cyclophosphamide was 125 mg/kg and the volume of exsanguination was 300 ml.

Hirota et al. (1995) induced **chemical peritonitis in rats** by applying 0.02 M HCl on the surface of the cecum or appendix and quantified the inflammation by measuring the extravasation of intravenously injected Evan's blue bound to albumin extracted from those tissues.

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H.3.2.2.3

Inhibition of Leukocyte Adhesion to Rat Mesenteric Venules In Vivo

PURPOSE AND RATIONALE

Reversible adherence of leukocytes to endothelium, basement membranes and other surfaces is an essential event in the establishment of inflammation. Their entry into tissues is controlled by the dynamic interaction between adhesion molecules expressed by these cells and the endothelium. White cells circulating in the blood have the tendency to adhere to the walls of blood vessels and this tendency is greatly increased in states of inflammation. Normally, when leukocytes collide with the vessel wall, the collision behaves elastically and the cells bounce off and back into the lumen. However, biochemical changes in inflamed tissues results in inelastic collisions of cells and an increase in their adhesion, thus initiating rolling of leukocytes along the endothelial surface. As adhesion further increases, rolling is slowed and may be followed by the cells coming to a complete stop and their migration out of the vessel. This can be observed by preparing a mesenteric venule of an anesthetized rat and following the flow and rolling of leukocytes by means of a microscope, thus allowing in-vivo studies. In this test procedure, adhesion of leukocytes, to the vessel wall, is artificially induced by the application of the formyl-methionyl peptide fMet-Leu-Phe (FMLP). Formyl peptides are released from bacteria and mitochondria of damaged tissue, so these peptides provide a specific signal marking the presence of invading bacteria or tissue damage. The density of FMLP receptors ranges from 10⁴ to 10⁵ per cell, depending on the cell type. Activation of leukocytes through this receptor results in rapid expression of preformed L-selectin (LECAM-1) on the cell surface which causes the cells to roll along the endothelial surface. LECAM-1 are very rapidly shed from the surface of leukocytes, however, and integrins take over to maintain further adhesion and migration into the tissue.

PROCEDURE

Sprague-Dawley rats are anesthetized by administration of Nembutal. The trachea, jugular vein and carotid artery are prepared free, the abdominal cavity is opened and a section of ileum is pulled out and draped over a heated microscope table. Prior to test compound administration, the number of spontaneous adhering leukocytes is counted, every 5 min, in a defined section of a venule (covered with paraffin oil) during a 30-min period (control). Blood pressure, body

temperature and velocity of blood flow are also registered. The test compound is administered via continuous infusion during the entire test procedure beginning at $t = -30$ min. Following the determination of control values for spontaneous adhesion, FMLP (f-Met-Leu-Phe, 10^{-4} M) is dripped twice ($t = -30$ min and $t = 0$ min) on the preparation and the number of adhering leukocytes is determined every 5 min over a 90 min period, beginning with the second application of FMLP ($t = 0$ min). Each test group consists of at least 10 animals. The mean leukocyte count of every rat prior to FMLP and/or test substance application is taken as the 100% value to obtain the baseline for further comparisons. The test compounds are dissolved in 0.9% NaCl shortly before application.

EVALUATION

Following the second topical application of FMLP (10^{-4} M), the number of adhering leukocytes in the mesenteric venule section is counted 30 min after the stimulus is given, and again at the end of the observation period of 2 h. The influence of a continuous i.v. infusion of the test drug is compared with the positive control group (FMLP stimulation, without drug).

MODIFICATIONS OF THE METHOD

A simple, rapid, *in vitro* assay for granulocyte adherence was developed by MacGregor et al. (1974). Heparinized whole blood is filtered through nylon fibers packed in Pasteur pipettes, and the percentage of granulocytes adhering was calculated.

Neutrophil adherence was tested *in vitro* by Burch et al. (1992). Human umbilical vein endothelial cells were plated at 5×10^4 cells/well into collagen coated culture plates and grown to confluence. Neutrophils were labeled with ^{51}Cr . Experimental agents were added to the neutrophils before their activation with FMLP. After 15 min incubation at 37°C , the non-adherent leukocytes were removed by gentle aspiration followed by a wash with saline. The adherent neutrophils were lysed by 1 N NaOH and the radioactivity was quantitated.

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H.3.2.2.4

Oxazolone-Induced Ear Edema in Mice

PURPOSE AND RATIONALE

The oxazolone-induced ear edema model as first described by Evans (1971) in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration.

PROCEDURE

Mice of either sex with a weight of 25 g are used. Before each use a fresh 2% solution of oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) in acetone is prepared. The mice are sensitized by application of 0.1 ml on the shaved abdominal skin or 0.01 ml on the inside of both ears under halothane anesthesia. The mice are challenged 8 days later again under anesthesia by applying 0.01 ml 2% oxazolone solution to the inside of the right ear (control) or 0.01 ml of oxazolone solution, in which the test compound or the standard is solved. Special pipettes of 0.1 ml or 0.01 ml are used. Groups of 10 to 15 animals are treated with the irritant alone or with the solution of the test compound. The left ear remains untreated. The maximum of inflammation occurs 24 h later. At this time the animals are sacrificed under anesthesia and a disc of 8 mm diameter is punched from both sides. The discs are immediately weighed on a balance. The weight difference is an indicator of the inflammatory edema.

EVALUATION

Average values of the increase of weight are calculated for each treated group and compared statistically with the control group. A 0.003% solution of hydrocortisone and a 1% solution of indomethacin were found to be active.

CRITICAL ASSESSMENT OF THE METHOD

The method is suitable for both steroidal and non-steroidal compounds as well as for the evaluation of various topical formulations.

MODIFICATIONS OF THE METHOD

Griswold et al. (1974) applied a 3% solution of oxazolone to the left paw of mice. The edema was assessed plethysmographically.

Various cutaneous models of inflammation for the evaluation of topical and systemic pharmacological agents have been discussed by Young and Young (1989).

Bailey et al. (1995) described a contact hypersensitivity model in mice for rank-ordering formulated corticosteroids. Male Swiss Webster mice were sensitized with 20 μ l of 2% oxazolone on the inner and outer aspects of each ear (10 μ l each side). Mice were challenged 7 days later with 2% oxazolone in acetone:olive oil (4:1) on both sides of the right ear. Animals were topically treated with corticosteroids or non-steroidal antiinflammatory drugs or 20 mg formulated corticosteroids immediately after challenge. The mice were sacrificed after 24 h and edema and myeloperoxidase activity were determined. Edema was measured by taking the weight of 6 mm trephine punch biopsies of the right and left ears. Inhibition was calculated from change in ear weight of control or drug treated ears versus placebo treated ears. Myeloperoxidase activity was assessed spectrophotometrically (Williams et al. 1983) on tissue homogenates. In a delayed-type hypersensitivity model animals were treated as in the contact hypersensitivity model, except the mice were sensitized with 40 μ l of 2% oxazolone in acetone:olive oil (4:1) on the unshaved inguinal areas.

Meingassner et al. (1997) studied anti-inflammatory activity using allergic contact dermatitis in **mice**, **rats** and **pigs**. Mice were sensitized on the shaved abdomen with 50 μ l of 2% oxazolone solution in acetone. After 7 days, they were challenged with 10 μ l of 2% (for topical testing) or 0.5% (for systemic testing) oxazolone on the inner surface of the right ears. Pinnal weight was taken as a measure of inflammatory edema 24 h after challenge. Female Sprague Dawley *rats* were sensitized by application of 80 μ l of 2,4-dinitrofluorobenzene solution applied in 20 μ l volumes to the inner surface of both ear lobes and to both shaved inguinal regions on day 1. Allergic contact dermatitis was elicited with 30 μ l of 0.5% 2,4-dinitrofluorobenzene applied to the test sites of \approx 15 mm in diameter on both shaved flanks on day 12. Animals were treated by gavage 2 h before and immediately after challenge. Dermatitis was evaluated by measuring the thickness of the lifted skin fold at the test sites with a spring-loaded micrometer. Domestic **pigs** were sensitized with 400 μ l of 10% 2,4-dinitrofluorobenzene applied to

four areas on both ears and groins. Challenge reactions were elicited 12 days later with 15 μ l of 2,4-dinitrofluorobenzene (1%) applied topically to test sites arranged in four craniocaudal lines on the dorsolateral shaved back (24 or 32 per pig). Test sites were treated twice either with 20 μ l solution of test compound or with \approx 50 mg of a cream formulation applied topically 30 min and 6 h after challenge. One day after challenge the test sites were visually evaluated for intensity and extent of erythema and induration.

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H.3.2.2.5**Croton-oil Ear Edema in Rats and Mice****PURPOSE AND RATIONALE**

The method has been developed primarily as a bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied steroids by Tonelli et al. (1965)

PROCEDURE

For tests in mice the irritant is composed as follows (v/v): 1 part Croton oil, 10 parts ethanol, 20 parts pyridine, 69 parts ethyl ether. For tests in rats the following mixture is prepared (v/v): 4 parts Croton oil, 10 parts ethanol, 20 parts pyridine, 66 parts ethyl ether. The standards and the test compounds are dissolved in this solution. For tests in mice male NMRI-mice with an weight of 22 g, for tests in rats male

Sprague-Dawley rats with a weight of 70 g are used. Ten animals are used for controls and each test group. The test compounds are dissolved in a concentration of 0.03 mg/ml to 1 mg/ml for mice and in a 3 to 10 times higher concentration for rats in the irritant solution. On both sides of the right ear 0.01 ml in mice or 0.02 ml in rats are applied. Controls receive only the irritant solvent. The left ear remains untreated. The irritant is applied under ether anesthesia. Four hours after application the animals are sacrificed under anesthesia. Both ears are removed and discs of 8 mm diameter are punched. The discs are weighed immediately and the weight difference between the treated and untreated ear is recorded indicating the degree of inflammatory edema. In the originally described method the ears are removed by sharp, straight scissors 6 h after application and weighed as total. The animals were sacrificed 48 h after topical administration and the thymus glands were removed, weighed and expressed as mg thymus/100 g body weight.

EVALUATION

The antiphlogistic effect can be determined by expressing the increase in weight of the treated ear as percentage of the weight of the contralateral control ear. The difference of both weights is divided by the weight of the contralateral ear times 100. Otherwise, the difference between both ears or excised discs is calculated as the average values for treated and control groups and the effect is evaluated by statistical methods. Concentration of 0.5 to 1 mg/ml hydrocortisone have been proven to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The method is useful for evaluation of anti-inflammatory topical steroids especially in the modification when thymus weight is determined simultaneously. The method also can be used for topically applied nonsteroidal antiphlogistics.

MODIFICATIONS OF THE METHOD

Wilhelmi and Domenjoz (1951) tested various drugs using Croton oil induced ear edema in mice and rabbits.

Tubaro et al. (1985) tested various anti-inflammatory drugs in the Croton oil test in mice. Granulocyte infiltration in plugs taken from the inflamed ears was assessed by measuring peroxidase activity.

Zentel and Töpert (1994) used Croton oil-induced ear edema in rats to evaluate topical corticosteroids. A plastic collar was fixed around the neck of Wistar

rats of either sex (160–200 g body weight) to exclude oral uptake of the compounds. Fifty μ l of 5% Croton oil in ethanol or ethanol alone were topically applied to both ears. In the treatment groups drugs were coapplied with Croton oil. Five h after treatment the animals were sacrificed by CO₂ gas and the ears removed. Edema formation was measured by the increase in wet weight.

Iwasaki et al. (1995) measured the inhibition of Croton oil-induced ear edema in Wistar rats by locally applied clobetasol-17-propionate, a synthetic glucocorticoid, and the influence of simultaneously applied RU 486.

Weirich et al. (1977) measured skin temperature, ear thickness and weight of excised punches after Croton oil induced edema in the ears of **white rabbits** and calculated phlogostasis values as the products of the percent reduction in skin temperature, auricular thickness and tissue weight in relation to controls. The authors recommended this model for the primary evaluation of topical anti-inflammatory agents.

Colorado et al. (1991) described an apparatus to measure Croton oil induced ear edema in mice using precisely reproducible pressure on the ear. The device allows to follow the time course of inflammation by repeated measurements.

Akiyama et al. (1994) studied staphylococcus aureus infection on experimental Croton oil-inflamed skin in mice. Staphylococcus aureus cells were inoculated on the surface of skin inflamed by application of Croton oil in cyclophosphamide-treated mice. Skin specimens were taken at 1, 3, 6, 12, and 24 h after inoculation and examined by microscopy. The staphylococcus aureus cells which attached to the surface of the skin immediately after inoculation had invaded the horny layer within 1 h. The cells gradually penetrated deeper into the epidermis. Application of corticosteroid ointments decreased the number of staphylococcus aureus cells in the lesions.

Anderson and Groth (1984) induced **toxic contact reactions** to Croton oil or dinitrochlorobenzene (DNCB), or **allergic contact reactions** to DNCB or oxazolone in guinea pig skin and tested the effect of various locally applied corticosteroids by macroscopic assessment and microscopic evaluation of cellular infiltrates.

Tarayre et al. (1984) used a 0.25% solution of **cantharidin** in acetone and applied 0.025 ml to one mouse ear. Two phases of inflammation were observed. After local application nonsteroidal drugs showed effects in the first phase only, whereas steroids influenced both phases.

De Young et al. (1987) induced ear inflammation in rats by intradermal injection of 10 ng **recombinant human interleukin-1 β** in 10 μ l of saline.

Maloff et al. (1989) injected 20 μ l of interleukin-1 solution into the left ear of mice and found a dose-dependent increase of ear thickness and myeloperoxidase activity which reached the maximum after 24 h. These effects were reduced by high doses of glucocorticoids but not by nonsteroidal anti-inflammatory drugs.

Chang et al. (1987) applied 4 μ g **tetradecanoyl phorbol acetate** and test drugs dissolved in acetone to the right ear of mice. Ear edema was calculated by subtracting the thickness of the left ear (vehicle control) from the right ear (treated ear).

De Young et al. (1989) examined the temporal patterns of edema and accumulation of the polymorphic nuclear cell marker enzyme myeloperoxidase following application of tetradecanoyl phorbol acetate to mouse ears. Topical and oral corticosteroids inhibited both edema and myeloperoxidase accumulation, whereby clobetasol propionate was more effective than fluocinolone and dexamethasone. Cyclo-oxygenase and lipoxygenase inhibitors were very effective against myeloperoxidase accumulation but were inactive or moderately active vs. edema.

Murakawa et al. (2006) studied the involvement of tumor necrosis factor- α in phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate- (TPA-) induced skin edema in mice.

Topical application of arachidonic acid to mouse ear has become a widely used test (Young et al. 1983, 1984; Opas et al. 1985; Crummey et al. 1987; Hensby et al. 1987; Tomchek et al. 1991) One mg arachidonic acid is applied to the right ear of mice and vehicle to the left ear of each animal. Drugs are topically applied in acetone to the ear 30 min prior to the arachidonic acid application. Ear swelling was measured using a caliper one hour after arachidonic acid.

Griswold et al. (1995) induced inflammation in mice by local application of arachidonic acid or phorbol ester. Besides ear thickness, myeloperoxidase and DNA content was measured.

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H.3.2.2.6

Paw Edema

PURPOSE AND RATIONALE

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil, sulfated polysaccharides like carrageenin or naphthoylheparamine. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Many methods have been described how to measure the paw volume by simple and less accurate and by more sophisticated electronically devised methods. The value of the assessment is less dependent on the apparatus but much more on the irritant being chosen. Some irritants induce only a short lasting inflammation whereas other irritants cause the paw edema to continue over more than 24 h.

PROCEDURE

Male or female Sprague-Dawley rats with a body weight between 100 and 150 g are used. The animals are starved overnight. To insure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again 3 and 6 h, and eventually 24 h after challenge.

Apparatus

Various devices have been developed for plethysmography of the paw. Winter et al. (1963) used mercury for immersion of the paw. A more sophisticated apparatus has been described by Hofrichter et al. (1969). Alpermann and Magerkurth (1972) described an apparatus based on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer. Webb and Griswold (1984) reported a sensitive method of measuring mouse paw volume by interfacing a Mettler DeltaRange top-loading balance with a micro-computer. Several authors used a commercially available plethysmometer from Ugo Basile, Varese, Italy (Damas and Remacle-Volon 1992; Braga da Motta et al. 1994; Legat et al. 1994; Griesbacher et al. 1994).

EVALUATION

The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal. Effectively treated animals show much less edema. The difference of average values between treated animals and control groups is calculated for each time interval and statistically evaluated. The difference at the various time intervals give some hints for the duration of the anti-inflammatory effect. A dose-response curve is run for active drugs and ED_{50} values can be determined.

MODIFICATIONS

Many agents can be used as irritants to induce paw edema in rats or mice. These are:

- 0.05 ml undiluted fresh egg white (Randall and Baruth 1976)
- 0.1 ml of 1% ovalbumin solution (Turner 1965)
- 0.1 ml of 1% formalin (Turner 1965)
- 0.1 ml of 0.2% carrageenan solution (Schönhöfer 1967) Introduction
- 0.1 ml of 1% carrageenan solution plus 100 ng PGE₂ or PGI₂ (Higgs et al. 1978; Portanova et al. 1996)
- 0.1 ml of 1 to 3% dextran solution (Turner 1965)
- 0.1 ml of 2.5% brewer's yeast powder suspension (Tsumuri et al. 1986)
- 0.1 ml of 0.5% β -naphthoylheparamine solution (Peterfalvi et al. 1966)
- 0.1 ml of 0.1% trypsin solution (Kalbhen and Smalla 1977)

- 0.1 ml of 0.1% collagenase solution (Souza Pinto et al. 1995)
- 0.1 ml of 0.1% solution of collagenase from *Clostridium histolyticum* (Legat et al. 1994)
- 0.1 ml of solution of 100 IU hyaluronidase (Dewes 1955, Kalbhen and Smalla 1977)
- 0.1 ml of complete Freund's adjuvant
- 0.05 ml of 0.02% serotonin solution (Kalbhen and Smalla 1977)
- 0.1 ml of 0.005% bradykinin solution (Damas and Remacle-Volon 1992)
- 0.1 ml of 0.1 mg/ml prostaglandin E2 (Nikolov et al. 1978)
- 0.1 ml of 2.0 µg/ml prostaglandin E2 (repeated injections, Willis and Cornelsen 1973)
- 0.1 ml of 1% concanavalin A solution (Lewis et al. 1976)
- 0.1 ml of 2.5% suspension of Aerosil
- 0.1 ml of 5% suspension of kaolin (Lorenz 1961; Wagner-Jauregg et al. 1962)
- 0.05 ml of bentonite gel (Marek 1980)
- 0.1 ml of nystatin 15000 units (Schiatti et al. 1970, Arrigoni-Martelli et al. 1971)
- 0.1 ml of 1% phytohaemagglutinin-P solution (Lewis et al. 1976)
- 0.01 ml of 0.5% adriamycin (mouse paw) (Siegel et al. 1980)
- 0.1 ml of 0.001–0.1% solutions of various phospholipases A2 (Cirino et al. 1989)
- 0.1 ml of 0.1% Zymosan solution (Gemmell et al. 1979)
- 0.1 ml of 0.05% anti-IgG solution (Gemmell et al. 1979)
- 0.1 ml of 2.5% mustard powder suspension (Tsumuri et al. 1986)
- 0.1 ml of solution containing 1 unit of cobra venom factor (Leyck and Parnham 1990)
- 0.05 ml of 0.02–0.2% sonic extract from *Porphyromonas gingivalis* (Griesbacher et al. 1994)
- 0.1 ml of 0.25% suspension of papaya latex (Gupta et al. 1994)

The edema induced by the various irritants lasts for different times such as a few hours after serotonin and up to 2 days after Aerosil or after kaolin. These irritants therefore are suitable to study not only the degree but also the duration of the anti-inflammatory action.

Standards

Depending on the irritant steroidal and nonsteroidal anti-inflammatory drugs have a pronounced effect in the paw edema test. With carrageenan as irritant doses of 50 to 100 mg/kg phenylbutazone p.o. have been found to be effective.

CRITICAL ASSESSMENT OF THE METHOD

The paw edema method has been used by many investigators and has been proven to be suitable for screening purposes as well as for more in depth evaluations. Dependent on the irritant steroidal and nonsteroidal anti-inflammatory drugs, antihistaminics and also, to a lesser degree, serotonin antagonists are active in the paw edema tests. Since so many different irritants have been used by the various investigators the results are often difficult to compare.

FURTHER MODIFICATIONS OF THE METHOD

Besides paw volume Shirota et al. (1984) determined the surface temperature of the inflamed paw in rats using a special cage with rolling rods.

Kunz et al. (2004) assessed protein patterns in lumbar cord during a zymosan-induced paw inflammation in rats employing a two-dimensional gel electrophoresis revealing a time-dependent breakdown of scaffolding proteins such as neurofilament light chain protein. A calpain inhibitor prevented inflammation-induced neurofilament light chain breakdown in the spinal cord and reduced hyperalgesia.

Brooks et al. (1991) used anesthetized **dogs** and demonstrated that a significant inflammatory response can be elicited in the dog paw by subcutaneous injection of carrageenan. The increase in paw volume can be quantitatively measured as a pressure change recorded via a water-filled balloon fixed against the paw with nonexpandable tape. Effective doses of nonsteroidal antiinflammatory drugs were closer to human therapeutic doses in dogs than in rats.

Oyanagui and Sato (1991) described an **ischemic paw assay in mice**. A commercial rubber ring (1 × 1 mm, $d = 42$ mm) was bound 14 times to the right hind leg of mice just above the articulation. After 20 min of ischemia, the rubber was cut off with scissors. Paw swelling was measured after another 20 min of natural blood recirculation.

Wirth et al. (1992) described a **thermic edema** which was induced in anesthetized Sprague-Dawley rats by immersing paws of the right and left hindlimb into water of 55°C. Immediately thereafter, the rats received the test drug (the bradykinin antag-

onist Hoe 140) intravenously. Paw volume was measured at regular intervals by plethysmography.

Braga da Motta et al. (1994) described drug modulation of **antigen-induced paw edema in guinea-pigs**. Male short-haired guinea pigs weighing 250–350 g received on day 0 a single dorsal s.c. injection of 1 ml of phosphate buffered saline containing 20 µg of ovalbumin, dispersed in 1 mg Al(OH)₃. The animals were boosted with a similar injection of antigen on days 14, 21, and 28. Thirty five days after the first injection of antigen or Al(OH)₃, the animals received an intraplantar injection of 0.5, 5, 50, or 200 µg ovalbumin, diluted in 100 µl of phosphate buffered saline. Edema was measured 2, 4, 6, 8, 24, and 48 h after the challenge.

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H.3.2.2.7

Pleurisy Test

PURPOSE AND RATIONALE

Pleurisy is a well known phenomenon of exudative inflammation in man. In experimental animals pleurisy can be induced by several irritants, such as histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes, and nonspecific irritants, like turpentine and carrageenan (Survey by DeBrito 1989). Carrageenan-induced pleurisy in rats is considered to be an excellent acute inflammatory model in which fluid extravasation, leukocyte migration and the various biochemical parameters involved in the inflammatory response can be measured easily in the exudate.

PROCEDURE

Male Sprague-Dawley rats weighing 220–260 g are used. The animal is lightly anaesthetized with ether, placed on its back and the hair from skin over the ribs of the right side is removed using animal clippers. The region is swabbed with alcohol. A small incision is made into the skin under the right arm between the seventh and eighth rib. The wound is opened and a further

shallow incision is made into the exposed intercostal muscle. 0.1 ml of 2% carrageenin solution is injected into the pleural cavity through this incision. The injection needs to be made swiftly to avoid the risk of injuring the lung. The wound is closed with a Michel clip.

One hour before carrageenan injection and 24 and 48 h thereafter, groups of 10 rats are treated with the standard or the test compound subcutaneously or orally. A control group receives only the vehicle of medication. The animals are sacrificed 72 h after carrageenin injection by ether inhalation. The animal is pinned on a dissection board with the forelimbs fully extended. An incision in the skin over the xiphosternal cartilage is made to free the cartilage from overlying connective tissue. The cartilage is lifted with a forceps and a small cut is made with scissors in the body wall below to gain access into the pleural cavity. One ml of heparinized Hank's solution is injected into the pleural cavity through this cut. The cavity is gently massaged to mix its contents. The fluid is aspirated out of the cavity using a pipette. This is made easier if the dissection board is raised to an angle of 45–60°; the contents then pool in the corners of the cavity. The aspirated exudate is collected in a graduated plastic tube.

EVALUATION

One ml (the added Hank's solution) is subtracted from the measured volume. The values of each experimental group are averaged and compared with the control group. ED_{50} values can be calculated using various doses. Several other parameters can be used:

- Measuring the white blood cell number in the exudate using a Coulter counter or a hemacytometer,
- Determination of lysosomal enzyme activities,
- Determination of fibronectin,
- Determination of PgE_2 .

CRITICAL ASSESSMENT OF THE METHOD

The pleurisy model has been accepted as a reliable method to study acute and subacute inflammation allowing the determination of several parameters simultaneously or successively. The activity of steroids as well as of non-steroidal drugs can be measured (Tomlinson et al. 1994; Harada et al. 1996).

MODIFICATIONS OF THE METHOD

The Evans blue-carrageenan-induced pleural effusion model has been proposed by Sancilio (1969, 1973) for screening of compounds with anti-inflammatory activity.

Meyers et al. (1993) tested the effect of treatment with interleukin-1 receptor antagonist on the development of carrageenan-induced pleurisy in intact and adrenalectomized rats.

Fröde et al. (2001, 2002) tested the effects of TNF- α and IL-1 β , IL-6, and IL-10 and their specific antibodies in the acute inflammatory response induced by carrageenan in a **mouse** model of pleurisy. Adult Swiss mice received a single intrapleural injection of 0.1 ml of sterile saline containing 1% carrageenan. As the inflammatory response caused by carrageenan in the pleural space of the mice exhibits a biphasic response, peaking at 4 h, characterized primarily by neutrophils, and at 48 h due mainly to mononuclear cells, both interval points were studied. On the day of the experiment, different doses of TNF- α , IL-1 β , IL-6 or IL-10 and their antibodies were injected into the pleural cavity of anesthetized mice. After 4 h or 48 h, the animals were sacrificed, the thorax was opened and the pleural cavity was washed with 1 ml of sterile PBS containing 20 IU/ml heparin. All animals had been injected 60 min previously with a solution of Evans blue dye (25 mg/kg, 0.2 ml, i.v.) in order to evaluate the degree of exudation into the pleural space. Leukocytes were counted and evaluated microscopically. The amount of dye was estimated by colorimetry.

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H.3.2.2.8

Granuloma Pouch Technique

PURPOSE AND RATIONALE

The method originally invented by Selye has been developed for screening by Robert and Nezamis (1957) using croton oil as irritant. An aseptic inflammation resulting in large volumes of hemorrhage exudate is elicited which resembles the subacute type of inflammation. Instead of croton oil carrageenan can be used as irritant.

PROCEDURE

Male or female Sprague-Dawley rats with a body weight between 150 and 200 g are used. Ten animals are taken for controls and for test groups. The back of the animals is shaved and disinfected. With a very thin needle a pneumoderma is made in the middle of the dorsal skin by injection of 20 ml of air under ether anesthesia. Into the resulting oval airpouch 0.5 ml of a 1% solution of Croton oil in sesame oil is injected avoiding any leakage of air. Forty-eight hours later the air is withdrawn from the pouch and 72 h later any resulting adhesions are broken. Instead of croton oil 1 ml of a 20% suspension of carrageenan in sesame oil can be used as irritant. Starting with the formation of the pouch, the animals are treated every day either orally or subcutaneously with the test compound or the standard. For testing local activity, the test compound is injected directly into the air sac at the same time as the

irritant. On the 4th or the 5th day the animals are sacrificed under anesthesia. The pouch is opened and the exudate is collected in glass cylinders. Controls have an exudate volume between 6 and 12 ml, which is reduced dose dependent in the treated animals.

EVALUATION

The average value of the exudate of the controls and the test groups is calculated. Comparison is made by statistical means. A clear dose response curve could be found by s.c. injection of 0.5, 1.0 and 2.0 mg hydrocortisone acetate/rat. Also doses of 1.5 mg/kg indomethacin were found to be active.

CRITICAL ASSESSMENT OF THE METHOD

The method has been very useful to estimate the potency of anti-inflammatory corticosteroids both after local and after systemic application. By injection of a depot-preparation and induction of the granuloma pouch after various time intervals up to 4 weeks the duration of action can also be determined (Vogel 1963, 1965).

MODIFICATIONS OF THE METHOD

Carrageenin was used to induce exudate formation (Boris and Stevenson 1965).

Bobalik and Bastian (1967) developed a modified granuloma pouch technique in which *Mycobacterium butyricum* (adjuvant) was used as phlogistic agent.

Moreno (1993) sensitized rats by subcutaneous injection of methylated bovine serum albumin emulsified in complete Freund's adjuvant one week prior to the preparation of air pouches which were reinflated 4 days later. Seven days after formation of the air pouches inflammation was induced in the pouches by injection of 1 mg methylated bovine serum albumin.

Martin et al. (1994) described an air pouch model in the 6-day-old rat by injection of carrageenan. Besides the usual parameters, leukocyte influx and the level of prostaglandin E₂ in the pouch exudate were measured.

In order to measure the effects of different classes of proteinase inhibitors, Karran and Harper (1995) studied collagen degradation in subcutaneous air pouches in rats. The air pouches were formed in the dorsal region and were inflamed 6 to 8 days later by injecting λ -type carrageenan. Degradation of ¹⁴C-collagen was followed in the inflammatory exudate fluid of the air pouches.

Sugio and Tsurufuji S (1981) re-evaluated the vascular constriction hypothesis as the mechanism of anti-inflammatory action of glucocorticoids. Rats were injected with 8 ml of air subcutaneously on the dorsal

surface under light ether anesthesia to make an oval air sac. After 24 h, 4.0 ml of 2% heat-sterilized solution of carrageenin in 0.9% NaCl solution was injected into the air sac (day 0). Drug effects were tested on day 7. Vascular permeability in the granuloma pouch was measured using ¹²⁵I-HSA and ¹³¹I-HSA. About 1 μ Ci of purified ¹²⁵I-HSA in 0.2 ml saline was injected into the femoral vein. After 30 min, 1.0 ml of the pouch fluid was withdrawn to measure the leakage of ¹²⁵I-HSA into the pouch fluid. After administration of the drug, about 1 μ Ci of purified ¹³¹I-HSA was injected into the femoral vein. After 30 min, 1.0 ml of the pouch fluid was again withdrawn to measure the concentration of ¹³¹I-HSA. The ratio of ¹²⁵I-HSA/¹³¹I-HSA was taken as an index of vascular permeability change induced by drug treatment.

Atkinson et al. (1962) implanted compressed pellets of carrageenin subcutaneously to rats and measured the effects of some anti-inflammatory substances on wet weight of the pellets.

Bowers et al. (1985) described a method to induce a **granuloma in the rat lung** by instillation of a 2% carrageenan solution into one lower lobe of the lung via the trachea. No respiratory impairment was noticed during this procedure.

Further phlogistic agents inducing specific inflammatory cascades such as zymosan (complement activation) or lipopolysaccharide (cytokine release) have been used for pharmacological evaluation of anti-inflammatory agents (Erdö 1994; Miller 1997).

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H.3.2.2.9

Urate-Induced Synovitis

PURPOSE AND RATIONALE

The importance of urate in gout and the deposition of sodium urate in gouty tophi is well known. Faires and McCarty (1962) reported that they themselves were the subjects for a study injecting 20 mg sodium urate crystal suspension in their own knee-joint. They experienced severe pain and prostration which resembled an acute gouty attack. Based on this experience they developed an experimental model in dogs for testing anti-inflammatory compounds (McCarty et al. 1963, 1966).

PROCEDURE

Preparation of Sodium Urate Crystals

0.4 g (0.01 Mol) sodium hydroxide pellets are dissolved in 400 ml distilled water in a glass beaker; 1.68 g (0.01 Mol) uric acid is added. The resultant opaque preparation is allowed to remain overnight at room temperature. The next morning, the crystals are harvested by decanting the supernatant solution and are then washed 3 times in cold saline, resuspended in saline and sterilized in an autoclave. Suspensions for injections are kept in rubber-stoppered, multi-dose vials containing 15 to 24 mg of urate per ml.

Unanesthetized healthy dogs weighing between 18 and 25 kg are used. They are trained to lie quietly on their backs in a dog cradle under light restraint. The

skin above one knee is shaved, disinfected and a sterile 21-gauge needle inserted into the joint. Slight aspiration produces a small amount of clear, viscous synovial fluid, indicating entry into the joint. The needle is left in place, a syringe containing the urate suspension is attached and volumes from 0.1 to 0.5 ml are injected into the joint (approximately 2–10 mg urate).

One hour before the injection of urate crystals the animals are treated with the test compound or the standard. Experiments are designed so that a pair of dogs is tested on each of 2 days. On the first day, only one dog receives the drug. One week later the opposite knee of each dog is injected, but the other dog is treated.

EVALUATION

A scoring system is adopted in which inflammatory symptoms ranging from tenderness, limping, occasional 3-legged gait to complete 3-legged gait are scored from 1+ to 4+.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that the experiment originally has been performed in human volunteers and that the method closely resembles pathological conditions in man, due to animal protection law conditions the method can be recommended only for special investigations.

MODIFICATIONS OF THE METHOD

Carlson et al. (1986) developed an automated microcomputer-based system for determining canine paw pressure quantitatively in the dog synovitis model.

According to Phelps et al. (1967) dogs are anesthetized and placed on their sides with the hind leg firmly fixed with tape so that the femur and tibia form a 90-degree angle. The knee is punctured with a needle. When a few drops of synovial fluid can be withdrawn indicating a correct puncture of the joint, 6–10 ml of saline are injected to distend the joint and a polyethylene catheter is inserted through the needle, which is then withdrawn. 0.5 ml of a 0.02% sodium urate suspension are injected into the joint. The catheter is attached to a pressure transducer. Constant pressure recordings can be taken during the acute phase of inflammation. Pressure changes are plotted against time, whereby each dog is compared with his own control. Treatment with nonsteroidal anti-inflammatory drugs, such as indomethacin, show a considerable reduction of intraarticular pressure.

Fujihira et al. (1971) injected the urate suspension into the knee joint of a hind leg of well trained Beagle dogs. They were placed on three weighing ma-

chines whereby both forelegs rested on one balance, and the hindlegs individually on other balances. In this way, the relative change of weight on each hindleg after intra-articular injection of urate suspension can be measured, indicating a decrease of weight in the injected leg, counterbalanced to the other leg. Time response curves could be found after non-steroidal anti-inflammatory drugs.

Rosenthale et al. (1972) found a long-lasting inflammatory effect of prostaglandins PGE₁ and PGE₂ after injection in the knee joint of dogs.

Schaible and Schmidt (1985) induced an acute experimental arthritis in the knee joint of anesthetized cats by intraarticular injection of a 4% kaolin suspension and recorded the activity of single fine afferent units from filaments of the saphenous nerve.

Perkins and Campell (1992) injected either sodium urate crystals or Freund's complete adjuvant into one knee of rats. The maximum tolerated pressure was determined with or without treatment by analgesic drugs after 18–24 h (urate injections) or 64–70 h (Freund's complete adjuvant).

Daniel and Jouvin (1984) induced inflammation of the guinea pig palatal mucosa by injection of a microcrystalline suspension of monosodium urate.

Botrel et al. (1994) induced chronic inflammation in the knee joint of Beagle dogs by intra-articular injection of Freund's complete adjuvant. Besides body temperature, differences in skin temperature, difference in stifle diameter, the vertical force exerted by the arthritic hind limb measured by a force plate was chosen as parameter.

Schött et al. (1994) induced monoarthritis in rats by injection of 300 µg carrageenan in 50 µl saline into the right tibio-tarsal joint. Weight bearing was found to be an objective measure of arthritic pain.

Carleson et al. (1996) induced acute inflammation in the temporomandibular joint of rats by intraarticular injection of substance P and measured neurokinin A, calcitonin gene-related peptide and neuropeptide Y in the perfusate.

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H.3.2.3

Methods for Testing the Proliferative Phase (Granuloma Formation)

H.3.2.3.1

Cotton Wool Granuloma

PURPOSE AND RATIONALE

The method has been described first by Meier et al. (1950) who showed that foreign body granulomas

were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenin.

PROCEDURE

Male Wistar rats with an average weight of 200 g are anaesthetized with ether. The back skin is shaved and disinfected with 70% ethanol. An incision is made in the lumbar region. By a blunted forceps subcutaneous tunnels are formed and a sterilized cotton pellet is placed on both sides in the scapular region. The pellets are either standardized for use in dentistry weighing 20 mg or pellets formed from raw cotton which produce a more pronounced inflammation than bleached cotton. The animals are treated for 7 days subcutaneously or orally. Then, the animals are sacrificed, the pellets prepared and dried until the weight remains constant. The net dry weight, i. e. after subtracting the weight of the cotton pellet is determined.

EVALUATION

The average weight of the pellets of the control group as well as of the test group is calculated. The percent change of granuloma weight relative to vehicle control group is determined.

CRITICAL ASSESSMENT OF THE METHOD

The method has been useful for evaluation of steroidal and nonsteroidal anti-inflammatory drugs. For testing corticosteroids, the test can be performed in adrenalectomized rats.

MODIFICATIONS OF THE METHOD

Bush and Alexander (1960) produced granulomata in rats by means of cotton-wool pellets which have been impregnated with carrageenin.

Tanaka et al. (1960) implanted filter paper pellets soaked with 7% formalin solution in rats.

Hicks (1969) implanted pellets impregnated with irritant substances, such as capsicum oleoresin.

Instead of cotton pellets, paper disks have been implanted (Tsurumi et al. 1986).

Roszkowski et al. (1971) immersed the cotton pellets in a 1% carrageenan solution, dried overnight and soaked in a 0.25 oxytetracycline solution before implantation.

D'Arcy and Howard (1967) induced a localized inflammatory reaction in the chorio-allantoic membrane of the chick embryo by the implantation of a sterile filter paper disc, followed by re-incubation *in situ* for 4 days.

Rudas (1960) described a method for quantitative evaluation of the granulation tissue formed in experimental wounds. Plastic rings were incorporated into the wounds on the back of rats inhibiting contraction of the wound edges and epithelialization of the wound. The growth of granulation tissue inside the rings was measured.

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H.3.2.3.2

Sponge Implantation Technique

PURPOSE AND RATIONALE

The sponge implantation technique was described first by Saxena (1960) for short term experiments but was used subsequently to study the formation of granulomata using long-term implantation.

PROCEDURE

Sponges used for implantation are prepared from polyvinyl foam sheets (thickness 5 mm). Discs are punched out to a standard size and weight ($10.0 \pm$

0.02 mg) using a 13 mm cork borer. The sponges are then soaked in 70% v/v ethanol for 30 min, rinsed four times with distilled water and heated at 80°C for 2 h. Prior to implantation in the animal, the sponges are soaked in sterile 0.9% saline in which either drugs, antigens or irritants have been suspended. Typical examples include 1% carrageenan, 1% yeast, 1% Zymosan A, 6% dextran, heat killed *Bordetella pertussis* (4×10^9 to 5×10^{10} organisms/ml) or 0.5% heat killed mycobacterium tuberculosis.

Sponges are implanted in female Wistar rats weighing 150–200 g under ether anesthesia. A 20 mm dorsal incision is made and the dermis separated from the underlying muscle layer by insertion of blunt forceps to form separate cavities into which sponges are inserted. Up to 8 sponges may be implanted per rat. The dorsal incision is closed with Michel clips and the animals are maintained at a constant temperature of 24°C.

For short term experiments, the animals are treated with test drug or standard once before implantation orally or subcutaneously. For long term experiments, the rats are treated daily up to 3 weeks.

EVALUATION

For estimation of the fluid phase of sponge exudates, e. g. protein content, enzyme levels and biological mediators such as prostaglandins as well as for leukocyte migration, the sponges are removed already after 9 h.

For studying the chronic phase of inflammation besides dry weight DNA, indicating cell content, hexosamine, indicating glycosaminoglycane content, and hydroxyproline, indicating collagen content, can be determined.

CRITICAL ASSESSMENT OF THE METHOD

The sponge implantation technique has been proven to be a versatile method which was used and modified by many investigators.

MODIFICATIONS OF THE METHOD

Boucek and Noble (1955) implanted polyvinyl sponges in rats, hamsters, rabbits and humans.

Holm-Pedersen and Zederfeldt (1971) implanted 2 cubes $10 \times 10 \times 10$ mm of cellulose sponge connected with a silk suture. After various implantation periods, the sponges were dissected free and the strength of the connection between the two parts of the sponge was determined after removal of the connecting suture.

Paulini et al. (1974, 1976) implanted polyester-polyurethane sponges which were inserted at both ends

of a 15 mm long PVC tube separated by a cotton wool plug.

Bonta et al. (1979) used polyether sponges measuring $4 \times 1.5 \times 0.5$ cm. A thin polyethylene cannula is inserted into a hole of the sponge and fixed with two stitches. After implantation of the sponge the cannula is pulled through a subdermal tunnel to a neck incision where about 1.5 cm is exteriorized and closed with a tube sealer. One ml of a 2% carrageenin solution is injected into the sponge via the cannula. To study the local effect of drugs, the test compounds can be injected together with the carrageenin. The drugs can be administered repeatedly at any time.

The cannulated sponge method was further modified by Bragt et al. (1980) using a subdermally implanted Teflon cylinder. This cylinder is provided with holes to ensure contact and exchange between the inner chamber and the surrounding tissue and with two cannulae allowing injection of material and withdrawal of exudate at any given stage of granuloma development.

Damas and Remacle-Volon (1992) implanted in rats sterilized polyester sponges which were removed after 4 h and weighed.

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H.3.2.3.3**Glass Rod Granuloma****PURPOSE AND RATIONALE**

The glass rod granuloma as first described by Vogel (1970) reflects the chronic proliferative inflammation. Of the newly formed connective tissue not only wet and dry weight, but also chemical composition and mechanical properties can be measured.

PROCEDURE

Glass rods with a diameter of 6 mm are cut to a length of 40 mm and the ends rounded off by flame melting. They are sterilized before implantation by boiling in water. Male Sprague-Dawley rats with an initial weight of 130 g are anaesthetized with ether, the back skin shaved and disinfected. From an incision in the caudal region a subcutaneous tunnel is formed in cranial direction with a closed blunted forceps. One glass rod is introduced into this tunnel finally lying on the back of the animal. The incision wound is closed by sutures. The animals are kept in separate cages. The rods remain *in situ* for 20 or 40 days. Treatment with drugs is either during the whole period or only during the last 10 or 2 days. At the end the animals are sacrificed under CO₂ anesthesia. The glass rods are prepared together with the surrounding connective tissue which forms a tube around the glass rod. By incision at one end the glass rod is extracted and the granuloma sac inverted forming a plain piece of pure connective tissue. Wet weight of the granuloma tissue is recorded. The specimens are kept in a humid chamber until further analysis. For measurement of the mechanical properties the specimens are fixed into the clamps of the Instron^(R) instrument allowing a gauge length of 30 mm. The load until break is recorded with a crosshead speed of 50 mm/min. In order to calculate tensile strength (N/mm²), the value of load at rupture (N) is divided by cross sectional area (measured as volume = wet weight divided by length). Finally, the granuloma tissue is dried and the dry weight is recorded. In addition, biochemical analyses, such as determination of collagen and glycosaminoglycans, can be performed.

EVALUATION

Several parameters can be determined by this method. Granuloma weight was reduced by corticosteroids depending on dose and time of administration and was also diminished after treatment with non-steroidal anti-inflammatory agents and lathyrogenic compounds. Furthermore, antiproliferative terpenoids reduced the granuloma weight. The mechanical pa-

rameters showed different results after these drugs indicating a different mode of action. Treatment with corticosteroids increased tensile strength. Only after long term treatment with toxic doses a decrease was found. Anti-inflammatory compounds, such as acetylsalicylic acid or indomethacin and antiproliferative terpenoids showed an increase of strength at medium and high doses.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to most other granuloma methods, the glass rod granuloma measures the late proliferative phase of inflammation. Since the newly formed connective tissue is not contaminated with the irritant biochemical analyses can be performed. The peculiar feature is the possibility to study the mechanical properties of newly formed proliferative connective tissue.

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H.3.3**Side Effects of Anti-inflammatory Compounds**

See Vogel (2006).

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H.4**Antipyretic Activity****H.4.0.1****General Considerations**

Treatment with antipyretics has been very important in the pre-antibiotic era. Nevertheless, for treatment of acute viral diseases and for treatment of protozoal infections like malaria reduction of elevated body temperature by antipyretics is still necessary. For anti-

inflammatory compounds, an antipyretic activity is regarded as a positive side effect. To evaluate these properties, fever is induced in rabbits or rats by injection of lipopolysaccharides or Brewer's yeast.

H.4.0.2

Antipyretic Testing in Rats

PURPOSE AND RATIONALE

The subcutaneous injection of Brewer's yeast suspension is known to produce fever in rats. A decrease in temperature can be achieved by administration of compounds with antipyretic activity.

PROCEDURE

A 15% suspension of Brewer's yeast in 0.9% saline is prepared. Groups of 6 male or female Wistar rats with a body weight of 150 g are used. By insertion of a thermocouple to a depth of 2 cm into the rectum the initial rectal temperatures are recorded. The animals are febrile by injection of 10 ml/kg of Brewer's yeast suspension subcutaneously in the back below the nape of the neck. The site of injection is massaged in order to spread the suspension beneath the skin. The room temperature is kept at 22–24°C. Immediately after yeast administration, food is withdrawn. 18 h post challenge, the rise in rectal temperature is recorded. The measurement is repeated after 30 min. Only animals with a body temperature of at least 38°C are taken into the test. The animals receive the test compound or the standard drug by oral administration. Rectal temperatures are recorded again 30, 60, 120 and 180 min post dosing.

EVALUATION

The differences between the actual values and the starting values are registered for each time interval. The maximum reduction in rectal temperature in comparison to the control group is calculated. The results are compared with the effect of standard drugs, e. g. aminophenazone 100 mg/kg p.o. or phenacetin 100 mg/kg p.o.

CRITICAL ASSESSMENT OF THE METHOD

The antipyresis test in rats in can be regarded as a classical method in pharmacology.

MODIFICATIONS OF THE METHOD

Stitt and Shimada (1991), Shimada et al. (1994) induced fever in rats by microinjecting 20 ng PGE₁ directly into one of the brain's circumventricular organs

of the rat known as the organum vasculosum laminae terminalis.

Luheshi et al. (1996) induced fever by intraperitoneal injection of 100 μg/kg lipopolysaccharide into rats and measured the inhibition of fever by interleukin-1 receptor antagonist.

Telemetry has been used to record body temperature in animals (Riley et al. 1978; Gallaher et al. 1985; Clement et al. 1989; Guillet et al. 1990; Kluger et al. 1990; Bejanian 1991; Watkinson et al. 1996; Miller et al. 1997).

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H.4.0.3

Antipyretic Testing in Rabbits

PURPOSE AND RATIONALE

Lipopolysaccharides from Gram-negative bacteria, e. g. *E. coli*, induce fever in rabbits after intravenous injection. Only lipopolysaccharide fractions are suitable, which cause after 60 min an increase of body temperature of 1°C or more at a dose between 0.1 and 0.2 µg/kg. In the rabbit, two maxima of temperature increases are observed. The first maximum occurs after 70 min, the second after 3 h.

PROCEDURE

Rabbits of both sexes and of various strains with a body weight between 3 and 5 kg can be used. The animals are placed into suitable cages and thermocouples connected with an automatic recorder are introduced into the rectum. The animals are allowed to adapt to the cages for 60 min. Then 0.2 ml/kg containing 0.2 µg lipopolysaccharide are injected intravenously into the rabbit ear. Sixty min later the test compound is administered either subcutaneously or orally. Body temperature is monitored for at least 3 h.

EVALUATION

A decrease of body temperature for at least 0.5°C for more than 30 min as compared with the temperature value before administration of the test compound is regarded as positive effect. This result has been found after 45 mg/kg phenylbutazone s.c. or 2.5 mg/kg indomethacin s.c.

CRITICAL ASSESSMENT OF THE METHOD

Measurement of body temperature in rabbits with polysaccharide induced fever is a more sensitive test than the yeast fever in rats. Furthermore, the method is used as a decisive test for the absence of pyrogens in parenteral drugs by several pharmacopoeias such as USP 23 (1955).

MODIFICATIONS OF THE METHOD

Cashin and Heading (1968) described a simple and reliable assay for antipyretic drugs in **mice**, using intracerebral injection of pyrogens.

Davidson et al. (1991) tested the effect of human recombinant lipocortin on the pyrogenic action of the synthetic polyribonucleotide polyinosini:polycytidylic acid in rabbits.

Yeast-induced pyrexia in **rats** has been used for antipyretic efficacy testing by Loux et al. (1982) and Cashin et al. (1977).

van Miert et al. (1977) studied the effects of antipyretic agents on fever and ruminal stasis induced by endotoxins in **conscious goats**.

Petrova et al. (1978) used turpentine-induced fever in rabbits to study antipyretic effects of dipyrone and acetylsalicylic acid.

Lee et al. (1985) studied the antipyretic effect of dipyrone on endotoxin fever of **macaque monkeys**.

Loza Garcia et al. (1993) studied the potentiation of chlorpromazine-induced hypothermia by the antipyretic drug dipyrone in anesthetized rats.

Shimada et al. (1994) studied the mechanism of action of the mild analgesic dipyrone preventing fever induced by injection of prostaglandin E₁ or interleukin-1β into the organum vasculosum terminalis of rat brain.

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Chapter I

Antiarthrotic and Immunomodulatory Activity¹

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¹With contributions to this and former editions by A. Huwiler, R.X. Raiss, R.R. Bartlett, and R. Schleyerbach.

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I.1 Anti-Arthrotic Activity

I.1.0.1 General Considerations

Multifactorial causes can lead to osteoarthritis (OA), and its pathogenesis is not clearly understood as yet. The main characteristics of OA are the slowly progressing deterioration of the articular cartilage, accompanied by intermitted painful inflammatory episodes, and a continuous subchondral bone remodeling, often resulting in osteophyte formation in non-weight-bearing joint areas. Because of the lack of innervation and vascularization of cartilage, the destruction of this specific tissue remains unnoticed until other joint compartments are involved such as synovial membranes, answering with reactive synovitis to cartilage debris, or mechanoreception changes in the underlying bone, or until the diminution of articular cartilage results in a radiographically detectable joint space narrowing.

Therefore, analgesic and anti-inflammatory therapy has been the major treatment for OA in the past, and NSAIDs still constitute 92% of the drugs used against OA. Intraarticular injections of corticosteroids are also applied, although with geographically variable emphasis. Nevertheless they are still considered a useful tool in severe cases. Both classes of drugs are now reviewed more carefully with regard to their potentially harmful effects on cartilage maintenance and chondrocyte function – which leads to a more critical approach in drug selection. So-called ‘chondroprotective’ drugs, mainly sulfated polysaccharides, played a certain role in the pharmacotherapy of OA in Central Europe and some Eastern countries, but failed to demonstrate clinical efficacy, and now have lost significance.

Anti-oxidative enzymes or drugs such as superoxide dismutase or diacerein are also considered to influence osteoarthrotic conditions. Recently, matrix metalloprotease-inhibitors (MMP-I), originally designed to inhibit tumor cell invasion, have shown promising results in counteracting the progressive enzymatic cartilage degradation, and some compounds are being developed for this indication. A further treatment gaining interest are derivatives of hyaluronic

acid, which are applied intra-articularly in a series of injections. Their mechanism, however, is not clear yet, and most preparations have been filed as ‘devices’ rather than ‘drugs’, claiming a viscosupplementation with anti-inflammatory, analgesic, and chondroprotective properties.

I.1.1 In Vitro Methods for Anti-Osteoarthrotic Activity

I.1.1.1 General Considerations

Since most of the drugs in use for OA were originally selected for other (e. g. arthritic) indications and only subsequently claimed to be effective in OA, they have not been primarily selected and optimized by *in vitro* assays specific for this condition. Thus, the indication lacks commonly agreed upon *in vitro* assays as well as clearly defined standard drugs to evaluate such models. Correspondingly, the variety of assay systems used to test compounds for their effect on cartilage maintenance and/or degradation is large, and the list below reflects this multitude.

The *in vitro* systems applied to assess drug effects upon chondrocytes range from homogenates of cartilage (Yu et al. 1991; Vignon et al. 1991; Zafarullah et al. 1992) over chondrocyte monolayers at different culture conditions and passages, suspensions of aggregated chondrocytes or clusters, cells cultured in or over an artificial matrix like agarose to cartilage explants and even organ cultures (Korver et al. 1989) like mouse patellas (Verschure et al. 1994). The species used in these studies vary over an equally wide range from mouse (Mohamed-Ali 1992), rat (Ismail et al. 1991; Seed et al. 1993; Seong et al. 1994; Srinivas et al. 1994), rabbit (Akatsuka et al. 1993; Collier, Ghosh 1991; Shimazu et al. 1993), dog (Venn et al. 1990), cattle, to human tissues (Bulstra et al. 1992; Green et al. 1995) derived from normal as well as osteoarthrotic conditions. The chondrocyte and cartilage explant culture systems used for several years are described in more detail below with special emphasis on comparability and standardization.

PURPOSE AND RATIONALE

Articular chondrocytes not only control the regular balance of matrix synthesis and degradation in healthy cartilage turnover, but are also regarded as key players in the enhanced degradation and finally reduced synthesis of matrix components in pathological conditions like OA. The two main cartilage constituents are col-

lagen type II fibrillar network, and proteoglycans attached to hyaluronic acid filaments, also termed aggregans. The latter are the more sensitive and the first ones to change in cartilage degradation. Therefore, the *in vitro* assays are performed mainly with chondrocytes, and the parameters measured focus on proteoglycan synthesis and/or degradation.

The exact mechanisms of cartilage pathophysiology are not yet elucidated, but enzymatic degradation involving metallo-proteinases are considered the main events. *In vitro*, interleukin-1 and retinoic acid induce enhanced matrix degradation as well as reduced matrix synthesis, as observed in OA. Their role in the actual disease process *in vivo*, however, remains obscure. They therefore are used rather as tools to induce a disease-relevant condition *in vitro* than being subject of direct pharmacological intervention. Thus, the *in vitro* assays described here are suitable to compare and select a variety of drugs for their effect upon the main biological activity of articular chondrocytes. To address the respective mechanisms of drug action, more specific follow-up assays like enzyme inhibition or cytokine release or inhibition tests should be applied.

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I.1.1.2

Modulation of Cellular Proteoglycan Metabolism

PURPOSE AND RATIONALE

In primary cultures, articular chondrocytes grown in an artificial matrix after digestion of the original bone, maintain their characteristic synthesis and turnover rate of cartilage matrix macromolecules for a long time. These metabolic processes can be influenced pharmacologically. In the following assay, compounds are tested for their effect upon the normal turnover of cartilage matrix by chondrocytes. The test is used to detect stimulation of matrix formation, but also to check for potential impairment of cartilage function. Specific matrix staining reveals the amount of newly formed matrix remaining around the cells at the end of treatment. Alternatively, incorporation of radiolabeled sulfate into the newly formed proteoglycans allows to quantitate the anabolic activity at the end of the experiment.

PROCEDURE

Reagents

A 1% (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

A 0.025% (w/v) solution of Collagenase type II, activity 242 U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 µg/ml gentamycin and 2.5 µg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at -20°C.

A 2% solution of low melting agarose from Seaplaque in 0.9% NaCl is prepared by heating in a microwave, and stored in a water bath at about 50°C.

A buffer of 25 mM sodium-acetate (2.051 g/l) with 0.4 M magnesium-chloride 6-hydrate (81.32 g/l) is prepared, and adjusted with acetic acid to pH 5.6.

A staining solution is prepared with 0.1 g of alcian-blue, obtained from Sigma, in 67.5 ml buffer, filtered, and supplemented with 10 ml of a 25% solution of glutaraldehyde.

An 8 M guanidinium-hydrochloride solution is prepared.

Tissue and Cell Preparation

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile scalpel, articular cartilage is then carefully removed from the underlying bone from all accessible cartilaginous regions and transferred into a sterile Ham's F12 solution at +4°C. The tissue is washed with Ham's F12 to remove adherent synovial fluid. The pieces are then transferred into a 150 ml trypsinizing flask, containing the pronase solution including the added serum, and incubated with gentle stirring for 1 h at 37°C and 95% humidity. The fluid is then removed, and the collagenase solution including the added serum is incubated with gentle stirring overnight. The resulting cell suspension is first filtered through a 90 µm and then a 50 µm Nylon filter and then centrifuged at 800 rpm for 10 min. Resuspension and washing is performed with HBSS and cells are counted and checked for vitality under the microscope using the Eosin staining. The vitality level should reach at least 95%. A cell suspension is prepared of 4×10^6 cells/ml Ham's F12 supplemented with 20% FCS.

To prepare the agarose cell cultures, 24 well plates are coated with 0.2 ml/well of a 1:1 mixture of the 2% agarose solution with preheated Ham's F12 and left at room temperature to gel. Then 0.1 ml/well

(0.2 ml/well for radiolabeling) of a 1:1 mixture of the above described cell suspension with the 2% agarose solution is added. Care has to be taken to maintain an even cell suspension, and not to overheat the cells during this procedure. After gel formation at room temperature, the multiwell plates are placed in the incubator, and 0.5 ml/well medium is added either 4 h later or the following day. The medium consists of Ham's F12 supplemented with 5% FCS and 25 µg/ml ascorbic acid, and is changed every second day.

Assay

The assay starts 5 days after cell preparation. Compounds are added to the medium in a final concentration of 10 µM with 6 to 8 replicae per compound, and added anew with each change of medium over a total period of 8 days. The concentration can be varied according to the expected potency of the drug studied. An untreated control group as well as standard compound groups are always included. As standard compound, pentosane polysulfate to check for matrix increase, or retinoic acid to cause matrix decrease, can be applied.

At the end of treatment, the medium is removed, the wells washed 3 × with 500 µl of medium without supplements, and 1 ml/well of the staining solution is added for 48 h. After removal of the supernatant, the following washing steps are performed for 10 min each:

- 3 × 500 µl/well 3% acetic acid,
- 1 × 500 µl/well 3% acetic acid in 25% ethanol,
- 2 × 500 µl/well 50% ethanol,
- 1 × 500 µl/well 70% ethanol.

With 500 µl/well of 8 M guanidinium hydrochloride solution, the bound stain is then extracted for 24 h at +4°C. After shaking the plates gently for 10 min, 100 µl/well of each supernatant is then transferred to round-bottom microtiter-plates, and the extinction photometrically assessed in the plate-reader at a wavelength of 610 nm.

EVALUATION

The extinction is expressed in percentage as staining density with the control values defined as 100%. Values $\geq 110\%$ are interpreted as stimulation of matrix formation, values lower than 80% as inhibition of matrix formation. Experiments with 8 wells/treatment usually exhibit a standard deviation below 7%.

CRITICAL ASSESSMENT OF THE TEST

The described method is suitable to compare up to 50 drugs in one experiment. The price for this is the limited quantification, as the staining is not strictly stoichiometric, and does not allow the distinction between matrix synthesis and degradation. For more detailed assessment, radiolabeling is the better choice. The limitation of these primary culture assays lies in the elaborate preparation and isolation of the chondrocytes. Several attempts to immortalize this differentiated mesenchymal cell type have resulted in the loss of cartilage-specific properties. A new cell line developed by MB Goldring (Green et al. 1995) might overcome this difficulty, but has not yet been reported to be modified pharmacologically.

MODIFICATIONS OF THE TEST

The agarose culture system for chondrocytes, originally described by Benya and Schaffer (1982), has been well characterized by Aydelotte et al. (1988, 1992), and the effects of different agarose densities have been studied by Verbruggen et al. (1990).

Instead of agarose gel cultures, some authors use 3D chondrocyte clusters in suspension (Bassleer et al. 1990, 1992; Henrotin et al. 1992), or suspensions over agarose (Archer et al. 1990), or embedded in collagen gels (Malemud et al. 1994).

Alternatively, encapsulation in alginate beads, either directly after isolation (Guo et al. 1989), even as primary culture for several months (Häuselmann et al. 1994), or after expansion in monolayers (Bonaventure et al. 1994), offers the opportunity to recover the chondrocytes later by depolymerization of the alginate.

Monolayers of articular chondrocytes can be used as well, but preferentially short-term (up to three days of culture), as under this culture condition chondrocytes tend to dedifferentiate to a fibroblast-like appearance and metabolic program. Authors using this modification are e. g. Kolibas, Goldberg (1989), Lane et al. (1992), and McCollum et al. (1991). The importance of culture conditions is addressed in the study by Seid et al. (1993), and that of culture duration in the paper by van der Kraan et al. (1992).

A dot blot assay by cuprolinic blue precipitation has been described by Jortikka et al. (1993), which is restricted to serum free conditions. Instead of matrix staining, radiolabeling can be applied as described in the next assay. In this case, the amount of cells should be doubled per well to assure sufficient label incorporation.

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1.1.1.3

Cellular Chondrocytic Chondrolysis

PURPOSE AND RATIONALE

In this assay a disease-relevant situation is achieved by adding interleukin-1 (IL-1) to articular chondrocytes grown in agarose gel. IL-1 suppresses proteoglycan (PG) synthesis as well as increases their degradation, and thus results in a process which is also observed in degradative joint diseases *in vivo*. This process is termed chondrocytic chondrolysis. The test is used to detect the potential interference of a drug with this pathological process. Effect upon PG synthesis is studied by radiolabeling at the end of the experiment, and measuring the amount of incorporated sulfate. The effect upon PG degradation, and its release from the cellular environment, can be examined by prelabeling with $\text{Na}_2^{35}\text{SO}_4$, and following the amount of released incorporated sulfate over time from the supernatant with each or every second medium change.

PROCEDURE

Reagents

A 1% (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

A 0.025% (w/v) solution of Collagenase type II, activity 242U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10% FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 $\mu\text{g}/\text{ml}$ gentamycin and 2.5 $\mu\text{g}/\text{ml}$ amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at -20°C .

A 2% solution of low melting Agarose from Seaplaque in 0.9% NaCl is prepared under heating and microwave application, and stored in a water bath at about 50°C .

Human recombinant interleukin-1 (IL-1) α or β from Genzyme is stored in aliquots at -20°C .

For radiolabeling, $\text{Na}_2^{35}\text{SO}_4$ is purchased from Amersham.

A 4 M and 8 M guanidinium-hydrochloride (Gu-HCl) solution is prepared.

Tissue and Cell Preparation

Tissue and cell preparations are performed as for the previous assay, except the initial number of cells/well should be 400000, corresponding to a cell-containing gel volume of 0.2 ml/well, and the medium added is supplemented with 10% FCS and 25 $\mu\text{g}/\text{ml}$ ascorbic acid. It is changed every second day.

Assay

The assay starts 6 days after cell preparation. Except for a control group, interleukin-1 α is added in a concentration of 3 U/well, and added anew with each consecutive medium change. Except for the IL-1-control group, compounds are added to the medium in a final concentration of 10 μM with 4 replicae per compound, and with the subsequent medium changes. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8 day treatment, the medium is replaced by medium containing 1 μCi $\text{Na}_2^{35}\text{SO}_4$ /well and incubated for 24 h. The supernatant is then removed, mixed 1:1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The multiwell plates with the remaining gels are deep frozen for at least 24 h to facilitate solubilization, thawed and then extracted with 500 $\mu\text{l}/\text{well}$ 8 M GuHCl supplemented with inhibitors. The content of each well is then centrifuged at 13,000 rpm for 30 min. After this step, the supernatant contains the matrix trapped around the cells. This is equally separated by a PD10 Sephadex column into free versus incorporated radiolabel. The probes containing the incorporated sulfate and derived from both fractions (medium and gel) are then mixed with scintillating fluid and assessed in a β -scintillation counter.

EVALUATION

Counts per minute (cpm) from medium and gel fraction are calculated and related to the total well content. They are added if total incorporation is measured, or left separately, in case the ratio between released versus retained label is of interest. The data are converted into percent incorporation, with the values of the untreated control group or those of the IL-1 control group serving as 100%.

CRITICAL ASSESSMENT OF THE TEST

This is a sensitive test, in which an adequate labeling protocol can provide detailed information. The time- and material-consuming separation of free from incorporated radiolabel at the end, however, limits the size of the experiments and number of compounds to be studied.

In both cellular tests drug effects should be checked for (anti)proliferative activity in a separate proliferation test.

MODIFICATIONS OF THE TEST

If the catabolic response to a drug is of more interest than the anabolic one, the radiolabeling can be shifted to a time point prior to treatment, and the release of incorporated sulfate into the supernatant will allow to follow the time course and amount of PG degradation. This prelabeling should not start earlier than 4 days after plating to assure a comparable matrix synthesis rate. Because IL-1 suppresses PG synthesis at a lower concentration than it stimulates its degradation, a double to triple amount of IL-1 should be used in this modification.

Instead of bovine, human chondrocytes can be used (Raiss et al. 1992). One should allow 4 days of adjustment in the agarose system before starting treatment with human cells. If available, human serum gives a higher PG synthesis rate than fetal calf serum (Oestensen et al. 1991). The heterogeneity of responses depends on the individual source and should be considered (Verbruggen et al. 1989).

When using human instead of bovine cells, the stimulation of degradation and inhibition of synthesis is more effective with IL-1 β than IL-1 α : a concentration of 0.1 U/ml results in a reduction of PG synthesis of ca 50% (Raiss et al. 1995). When incubating with radiolabeled sulfate, the exposure time should be doubled to 48 h to yield sufficient incorporation.

To exclude direct interference of a drug with interleukin-1, all-trans retinoic acid can be used instead of this cytokine.

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I.1.1.4**Cartilage Explant Chondrolysis****PURPOSE AND RATIONALE**

Chondrocytes vary in their metabolic activity and cytokine response depends on the relative location within the joint (superficial vs deep, weight-bearing vs non-weight-bearing, etc). Therefore, the cellular assays are a homogeneous mixture of an otherwise heterogeneous cell population. Two reasons suggest a verification of the cellular results obtained in tissue culture assays: First, chondrocytes are more reactive after isolation compared to those in tissue culture, which may lead to false positive results. Second, intact cartilage matrix acts as barrier for certain compounds of high molecular size and fixed charge, so that they may not reach their target cells. Therefore, explant assays are recommended as a follow-up to the cellular tests.

PROCEDURE**Reagents**

Ham's F12 medium is supplemented with 50 μ g/ml gentamycin and 2.5 μ g/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at -20°C .

Human recombinant interleukin-1 (IL-1) α or β from Genzyme is stored in aliquots at -20°C .

For radiolabeling, Na₂³⁵SO₄ is purchased from Amersham.

A 4 M and 8 M guanidinium-hydrochloride (Gu-HCl) solution is prepared.

Tissue Preparation

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile punch (as used for obtaining skin biopsies) full thickness disks of cartilage are obtained from all accessible cartilaginous areas and their wet weight is assessed. In each well, 1 ml of medium is added, consisting of Ham's F12 supplemented with 10% FCS and 25 μ g/ml ascorbic acid, and approximately 30 mg wet

weight of cartilage are transferred corresponding to 3 discs of 4 mm diameter.

Assay

The assay is started 1 to 2 days after tissue preparation. Except for a control group, interleukin-1 α is added in a concentration of 8 U/well, and with each of the following medium changes (every second day). Except for an IL-1-control group, compounds are added to the medium in a final concentration of 10 μ M with 6 to 8 replicae per compound, which are also added with each medium change. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8 day treatment, the medium is replaced by a medium containing 15 μ Ci Na₂³⁵SO₄/well and incubated for 24 h. The supernatant is removed, mixed 1:1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The explants are washed three times with Ham's F12 at +4°C, and extracted with 1 ml/well 4 M GuHCl supplemented with inhibitors for 48 h, and then a second time with 0.5 ml/well for 24 h. Both fractions are mixed, and separated with a PD10 Sephadex column into free versus incorporated sulfate. The samples containing the incorporated sulfate derived from medium as well as explant extraction are then mixed with scintillating fluid and assessed in a β -scintillation counter.

EVALUATION

Counts per minute (cpm) from medium and explant fractions are calculated related to mg wet weight of cartilage of the respective wells. They are either added if total incorporation is measured, or left separately, in case the ratio between released versus retained matrix is of interest. The data are converted into percent incorporation in comparison with the values of the untreated control or of the IL-1 control group serving as 100%.

CRITICAL ASSESSMENT OF THE TEST

Punched discs of similar size standardize the surface/volume ratio, and give more reproducible results than chips of cartilage obtained by scalpel dissection. A disadvantage is the greater amount of cartilage needed, which makes it unsuitable for human tissue obtained from joint replacement surgery. When using human tissue, interpretation and comparison of results should be restricted to the same source: striking differences occur between specimens from surgery or post-mortem, between different joints (hip vs knee), and different ages of the donor (young or adolescent vs

35 years and older), as well as different stages of severity and the duration of degenerative joint diseases in cases of surgical specimens.

MODIFICATIONS OF THE TEST

The effect of serum concentrations on proteoglycan synthesis has been studied by McQuillen et al. (1986), and the effect of different concentrations of DMSO and glycerol, of importance for cryopreservation, has been examined on human fetal hip cartilage by Yang and Zhang (1991).

Some authors (Nixon et al. 1991) use bovine nasal septum as cartilage source, but the convenient homogeneity and mass of this tissue is outweighed by a matrix composition and biomechanical properties clearly distinct from articular cartilage.

Several authors use human cartilage from joint replacement surgery (e. g. Pelletier et al. 1989; Pelletier, Martel-Pelletier 1989), and some compare drug effects upon visually normal cartilage to those with fibrillated or osteoarthritic cartilage (Lafeber et al. 1992, 1993; Verbruggen et al. 1989, 1990).

A step towards organ culture represents the culture of full thickness cartilage with subchondral bone, cultured for 24 h on moist lens tissue (Chayen et al. 1994).

Bordji et al. (2000) published evidence for the presence of peroxisome proliferator-activated receptor (PPAR) α and γ and retinoid Z receptor in cartilage.

Bondeson et al. (2006) established a model of cultures of synovial cells from digested osteoarthritis synovium derived from patients undergoing knee or hip arthroplasties.

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I.1.1.5

Influence on Matrix Metalloproteases

PURPOSE AND RATIONALE

Matrix metalloproteases (MMPs) form a multigene family of more than 20 secreted and membrane-tethered zinc-dependent endopeptidases, which are classified according to their structures and substrate specificities (Nicholson et al. 2005). Members of the family include: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11, -12) and membrane-type matrix metalloproteases (MT1-MMP to MT6-MMP). They are regulated by natural inhibitors, such as α_2 -macroglobulin and the tissue inhibitors of metalloproteases (TIMPs). The catalytic domains of the MMPs have an ellipsoid shape with a small active cleft. This cleft contains the catalytic zinc ion, which is essential for catalysis. MMPs can degrade all components of the extracellular matrix and have been implicated in a number of pathological conditions. They are produced in response to the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) and are found in excess in the arthritic joint (Borkakoti 1998, 2004; Close 2001; Rosenblum et al. 2003; Mott and Werb 2004). MMPs belong to the metzincin superfamily of metalloproteases, which also includes astacins, ADAMs (proteins with a disintegrin and metalloprotease domain) and ADAM-TS (an ADAM with a thrombospondin-like motif). Several trials are ongoing to find inhibitors

of MMPs for treatment of rheumatism and osteoarthritis (Levin et al. 1998; Bigg and Rowan 2001; Martel-Pelletier et al. 2001; Matter et al. 2002; Nelson et al. 2002; Aranapakam et al. 2003; Skotnicki et al. 2003; Zask et al. 2003; Matter and Schudok 2004; Skiles et al. 2004). Besides these indications, metalloprotease inhibitors may be useful in treatment of other diseases, such as chronic obstructive bronchitis, atherosclerosis, inflammatory bowel disease, and cancer (Chang and Werb 2001; Whelan 2004).

Cauchard et al. (2004) tested the activation of latent transforming growth factor 1 and inhibition of matrix metalloproteinase activity by a thrombospondin-like tripeptide linked to elaidic acid.

PROCEDURE

In Vitro Assays

The influence of test substances on recombinant MMP-2, MMP-3, and MMP-9 activity was determined using the fluorescent quenched substrate Mca-Pro-Leu-Gly-Leu-Dpa-Leu-Ala-Arg [where Mca = (7-methoxy-coumarin-4-yl) acetyl and Dpa = 3-(2',4'-dinitrophenyl)-L-2,3-diaminopropionyl]. Each enzyme was active site-titrated using a standard preparation of human recombinant TIMP-1 for MMP-1, and TIMP-2 for MMP-2 and TIMP-9 for MMP-9. Then, 200 pM MMP-2 or MMP-9, 400 pM of MMP-3 or 3 nM of MMP-1 was mixed with increasing concentrations of test substances in a 50 mM HEPES buffer, pH 7.6, containing 150 mM NaCl and 5 mM CaCl_2 and the assay was initiated by adding 2 μM of substrate. The rate of substrate hydrolysis was linear up to 30 min at 22°C. The reaction was stopped by adding 10 mM EDTA. The rate of the reaction was measured in triplicate for each test drug concentration examined using Perkin-Elmer LS50B spectrofluorometer with excitation and emission wavelength of 325 and 375 nm, respectively. Less than 5% of the substrate was hydrolyzed during the rate measurements.

EVALUATION

IC₅₀ (μM) values were determined by plotting V_i/V_0 , where V_i is the rate of substrate hydrolysis in the presence of inhibitor, V_0 is the rate in its absence, as a function of test drug concentrations and non-linear regression analysis.

MODIFICATIONS OF THE METHOD

Similar procedures were used in the following studies:

Lewis et al. (1997) to study an orally active collagenase inhibitor;

Billingham et al. (2000) and Dahlberg et al. (2000) to compare the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1;

Berton et al. (2001) to study the inhibition of matrix metalloproteinase activities by long-chain fatty acids; and

Matter et al. (2002) to investigate structure–activity relationship of tetrahydroisoquinoline-3-carboxylate-based matrix-metalloprotease inhibitors.

Bottomley et al. (1997) studied the inhibition of bovine nasal cartilage degradation by selective matrix metalloprotease inhibitors.

The potency of the inhibitors against human collagenase 1 (MMP-1), stromelysin (MMP-3), gelatinase B (MMP-9), and aggrecan metabolism was tested. Medium from human dermal fibroblasts (CCD45) cultured in the presence of recombinant human IL-1 α (25 ng/ml) was used as source of collagenase. Collagenase activity was determined by measuring the degradation of ^{14}C -labeled collagen fibrils. Human prostromelysin-1 was antibody-affinity purified from conditioned human fibroblast culture medium. Progelatinase B was purified by gelatin-agarose affinity chromatography from human neutrophils. Both prostromelysin and procollagenase B were activated by treatment with trypsin. Stromelysin and gelatinase B activities were determined by measuring the cleavage of the fluorogenic substrate Mca-Pro-Lys-Pro-Leu-Gly-Leu-Dpa-NH $_2$; assays were performed using 50 mM borate buffer/1 mM CaCl $_2$ containing 0.05% Brij-35, at a substrate concentration of 2 μM . Assays were started by the addition of enzyme to a mixture of substrate and an inhibitor, and incubated for 4 h at 37°C; the assay was stopped by addition of acetic acid to a final concentration of 0.17 M. The fluorescence of the product Mca-Pro-Lys-Pro-Leu-Gly (λ_{ex} 325 nm; λ_{em} 395 nm) was measured with a Hitachi F-4500 fluorescence spectrophotometer.

A similar method was used by Reichelt et al. (2002) for design, synthesis, and evaluation of matrix metalloprotease inhibitors bearing cyclopentane-derived peptidomimetics as P1' and P2' replacements.

Perlman et al. (2003) found that IL-6 and matrix metalloproteinase-1 are regulated by the cyclin-dependent kinase inhibitor p21 in human synovial fibroblasts.

A fluorescent screening assay for collagenase using collagen labeled with 2-methoxy-2,4-diphenyl-3(2H)-furanone was recommended by O'Grady et al. (1984).

Zhang et al. (2004) identified and characterized a dual tumor necrosis factor- α -converting enzyme

(TACE)/matrix metalloprotease inhibitor for the treatment of rheumatoid arthritis.

Assay of TNF- α Converting Enzyme (TACE)

A synthetic peptide of pro-TNF- α containing the minimal TACE cleavage sequence, Abz-LAQAVRSSSR-Dpa, was used as substrate. A segment of the extracellular portion of the human TACE that comprises the catalytic domain, the disintegrin domain, the epidermal growth factor-like domain, and the Crambin-like domain was used. The protein was expressed in Chinese hamster ovary (CHO) cells and purified by nickel-nitrilotriacetic acid and preparative size exclusion chromatography to near homogeneity. Compounds were tested for their ability to inhibit the cleavage of the substrate by the purified enzyme in a fluorescence-based fluorescence resonance energy transfer (FRTE) assay. The human TACE protein (1 $\mu\text{g}/\text{ml}$) was pretreated with the inhibitors at various concentrations for 10 min at room temperature. The reaction was initiated by the addition of pro-TNF- α peptide to the TACE protein, and the increase in fluorescence was monitored at an excitation wavelength of 320 nm and emission wavelength of 420 nm over a period of 10 min (Jin et al. 2002).

MMP Enzymatic Assays

A continuous assay was used in which the substrate is a synthetic peptide containing a fluorescent group (7-methoxycoumarin), which is quenched by energy transfer to a 2,4-dinitrophenyl group. When the peptide was cleaved by MMPs, an increase in fluorescence was observed. The substrate used was 7-methoxycoumarine-PQGL-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-AR-OH. The assays were carried out at room temperature in a buffer containing 50 mM HEPES, pH 7.4; 100 mM NaCl, 5 mM CaCl $_2$, and 0.005% Brij-35 (Knight et al. 1992). The enzyme reaction was initiated by adding the substrate to a final concentration of 20 μM . The initial rate of the cleavage reaction was determined immediately after substrate addition.

Sadowski and Steinmeyer (2001) studied the effects of non-steroidal anti-inflammatory drugs and dexamethasone on the activity and expression of MMP-1, MMP-3 and tissue inhibitor of metalloproteinases-1. Bovine chondrocytes were cultured in alginate gel beads. Cells were treated with IL-interleukin 1 α in the presence of vehicle or drugs at various concentrations. After 48 h mRNA expression of MMP-1, MMP-3, and the tissue inhibitor of metalloproteinases (TIMP-1) was analyzed by RT-PCR-ELISA. The protein synthe-

sis of TIMP-1 and MMP-3 was determined by immunoprecipitation. The activity of enzymes and inhibitors was measured by functional assays (Yoshioka et al. 1987; Steinmeyer et al. 1998).

The synthesis and biological activity of **TACE-inhibitors** are described by Beck et al. (2002), by Letavic et al. (2002, 2003) and by Tsukida et al. (2004).

Downs et al. (2001) used an **ELISA** for analysis of collagenase-cleavage of type II collagen to the C-terminal neopeptide. Valleala et al. (2003) described an ELISA assay for MMP-9 (gelatinase B).

Microtiter plates were coated with 100 μ l of 5 μ g/ml MMP-9-specific monoclonal antibody (TNO-S22.2) in PBS overnight at 4°C. After three washes in PBS containing 0.05% Tween 20 (PBS-T), 100 μ l of purified MMP-9 or cell supernatant was added. After overnight incubation at 4°C, the plates were washed and incubated for 1 h at 37°C with 100 μ l of biotin-labeled anti-MMP-9 polyclonal antibody (TNO-B21) diluted in PBS-T/EDTA + 0.1% casein (PBS-T/DTA/C) (0.8 μ g/ml). After washing, bound polyclonal antibody was assessed by incubation with 100 μ l of avidin/HRP at 1:10,000 in PBS-T/EDTA/C. Non-bound conjugate was washed away after 1 h at 37°C, and the chromogene 3,3',5,5'-tetramethyl benzidine in the presence of H₂O₂ was added. The reaction was stopped after 20 min with 2M H₂SO₄ and the absorption was measured at 450 nm in a Titertek Multiscan spectrophotometer.

Peppard et al. (2003) developed an assay suitable for high-throughput screening to measure matrix metalloproteinase activity.

Sabatini et al. (2005) studied the effect of inhibition of matrix metalloproteinases on cartilage loss *in vitro* and in a guinea pig model of osteoarthritis.

Zymography was used by several authors to measure the activity of metalloproteinases (Maquoi et al. 1998, 2002; Hattori et al. 2002; Sartor et al. 2002; Sato et al. 2002; Kaji et al. 2003; Kerkvliet et al. 2003; Kim and Kim 2004; Liu et al. 2004; Martin-Chouly et al. 2004; Naqvi et al. 2005).

Samples were subjected to electrophoresis on a 4.5% acrylamide stacking gel/7% acrylamide separating gel containing 1 mg/ml gelatin, in the presence of sodium dodecyl sulfate, under non-reducing conditions. After electrophoresis, gels were washed twice with 2.5% Triton X-100, rinsed with water, and incubated at 37°C overnight in 50 mM Tris, 5 mM CaCl₂, 2 mM ZnCl₂, pH 8.0. The gels were stained with Coomassie brilliant blue and destained in a solution of 25% ethanol and 10% acetic acid. Gelatinase

activities appeared as clear bands against a blue background. To determine the inhibition profile of the enzyme activities, gels were incubated in the presence of one of the following inhibitors in the activation buffer: 10 mM EDTA as an inhibitor of MMPs, or 10 mM PMSF (phenylmethylsulfonyl fluoride) as an inhibitor of serine proteases. The molecular weight of gelatinolytic bands was estimated using recombinant protein molecular weight markers. Images of zymograms were acquired with the Gel Doc 1000 Gel Documentation System. The amount of enzyme was quantified by measuring the intensity of the negative bands using a densitometer analyzer with Quantity one software. Results were expressed as arbitrary units of relative intensity.

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I.1.1.6

Aggrecanase Inhibition

PURPOSE AND RATIONALE

Degenerative joint diseases are characterized by cartilage extracellular matrix degeneration, where loss of aggrecan, an aggregating proteoglycan, is an early event in the destruction of articular cartilage. Aggrecan, a multidomain proteoglycan, is a major component of cartilage and provides compressive resistance to articular cartilage. During the early stages of osteoarthritis, and then throughout the disease, there is increased loss of glycosaminoglycan-rich aggrecan fragments via proteolysis attributable to “aggrecanase” activity.

Aggrecanase-1 and -2 are members of the ADAMTS (a disintegrin and metalloproteinase possessing thrombospondin domain) family of zinc-containing metalloproteinases, responsible for the cleavage of the aggrecan interglobular domain (IGD) at the Glu³⁷³-Ala³⁷⁴ peptide bond, a unique site untouched by any previously identified enzyme (Arner et al. 1998; Abbaszade et al. 1999; Arner et al. 2002; Little et al. 1999; Tortorella et al. 1999, 2001; Arner 2002; Patwari et al. 2005; Stanton et al. 2005; Wight 2005; Song et al. 2007).

Inhibition of aggrecanase may impart overall cartilage protection and offer a potential therapy to alter the progression of osteoarthritis. Besides of endogenous inhibitors of aggrecanase (Bonassar et al. 1997; Sandy et al. 1998; Hashimoto et al. 2001, 2004; Little et al. 2002a; Malfait et al. 2002; Munteanu et al. 2002; Gendron et al. 2003; Pratta et al. 2003; Tortorella et al. 2004); also synthetic inhibitors are described (Bottomley et al. 1997; Munteanu et al. 2000; Little et al. 2002b; Sabatini et al. 2002, 2005; Sawa et al. 2002; Wada et al. 2002; Cherney et al. 2003; Vankemmelbeke et al. 2003; Noe et al. 2004; Liacini et al. 2005).

Xiang et al. (2006) described the synthesis and biological evaluation of biphenylsulfonamide carboxylate aggrecanase-1 inhibitors.

PROCEDURE

Bovine carpal joints were obtained from young (1- to 2-week-old) animals. Full-depth articular cartilage plugs were harvested using a cork borer and then sliced on a custom dice to generate individual disks 6 mm wide, 1 mm thick, and 30 mg in weight.

Cartilage explants were cultured at 37°C for 5 days in a humidified atmosphere of 5% CO₂ in air in cartilage explant media (CEM) consisting of Dulbecco's modified Eagle's medium containing 1% an-

timycotic/antibiotic, 2 mM glutamine, 10 mM HEPES, and 50 µg/ml of ascorbate (all from Sigma, St. Louis, Mo., USA). The explants were washed with CEM and one weighed disk per well was placed in a 96-well culture dish with 0.2 ml medium and six to eight replicates per treatment and cultured for 3 days in the presence or absence of recombinant human IL-1 α (rhIL-1, 5 ng/ml, Sigma) and the presence or absence of small molecule compound. Media were replaced every day. The proteoglycan content in the medium was measured as sulfated glycosaminoglycan (GAG) by a colorimetric assay (Farndale et al. 1982) using dimethylmethylene blue (DMMB) and chondroitin sulfate C from shark cartilage (Sigma) as a standard. Treatment of cartilage with IL-1 results in the induction of catabolic enzymes including aggrecanases that degrade cartilage matrix proteoglycan. The cleaved proteoglycan is released from the matrix into the media. Addition of the compound together with IL-1 to the cartilage results in a decrease of proteoglycan release, indicating the inhibition of proteoglycan degradation. During this early phase of proteoglycan degradation, aggrecanases are the predominant catabolic enzymes that cleave aggrecan with no significant role of other MMPs (Pratta et al. 2003).

EVALUATION

Measured proteoglycan was expressed per weight of cartilage. IC₅₀ values were calculated.

MODIFICATIONS OF THE METHOD

Miller et al. (2003) reported a microplate assay specific for the enzyme aggrecanase. Peppard et al. (2003) developed a high-throughput screening assay for inhibitors of aggrecan cleavage using luminescent oxygen channeling (AlphaScreen).

A simplified assay was reported by Kashiwagi et al. (2001).

Activities of ADAM-TS4 and ADAM-TS5 were measured by incubating enzyme with purified bovine aggrecan (500 nM) in 100 µl of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 10 mM CaCl₂, for 2 h at 37 °C and terminating the reaction with 10 mM EDTA. The digestion products were then deglycosylated with chondroitinase ABC (0.1 units/10 µg aggrecan) and then with keratinase (0.1 units/10 µg aggrecan) and keratinase II (0.002 units/10 µg aggrecan) for 2 h at 37 °C in 0.1 M Tris-HCl, pH 6.5, containing 50 mM sodium acetate. The enzymatically treated products were analyzed by Western blotting using BC-3 antibody or an antibody against the GELE¹⁴⁸⁰ neoepitope (Tortorella et al. 2000). To de-

termine apparent inhibition constant $K_{i(app)}$ values for inhibitors against the aggrecanases, ADAM-TS4 or ADAM-TS5 (at a final concentration of 50 pM) was incubated with various concentrations of the inhibitor in 44 µl of the above buffer at room temperature for 30 min and then a solution of bovine aggrecan (5.5 µl) was added. The reaction products were detected with anti-GELE¹⁴⁸⁰ antibody. The concentrations of ADAM-TS4 and ADAM-TS5 were confirmed by titration with recombinant N-TIMP-3.

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I.1.2

In Vivo Methods for Anti-Osteoarthritic Activity

I.1.2.1

General Considerations

The current availability of animal models of osteoarthritis (OA) for pharmacological assessment is impeded in several aspects: Firstly, the difficulty to address a generally slow progression of cartilage destruc-

tion and deterioration of joint function as encountered in human OA with an animal model achieving sufficient similarity to human pathology in an acceptable time frame. Secondly, the discrepancy between the need of mild, reversible pathological changes, which can be modified therapeutically, and the paucity of reliable parameters with which to determine normal versus disease stages with a gradable range large enough to assess drug effects. This includes also the lack of validation (clinically as well as in animal models) of noninvasive methods to assess disease progression. Thirdly, the lack of a true disease-modifying standard drug with which to validate the pharmacological effects in respect to the predicted clinical outcome.

This results in a situation that animal models closer to human pathology like the spontaneous OA in the Hartley strain of guinea pigs or the surgically inflicted joint instability in the Pond-Nuki dog model are too elaborate to be used routinely for drug selection. On the other hand, models like the chymopapain-induced cartilage degradation in rabbits, are suitable to study drugs, but are limited in their predictive value.

Some progress can be expected from the use of genetically modified animals (Rintala et al. 1997; Serra et al. 1997; Ameye and Young 2002; Han et al. 2002; van Lent et al. 2002; Johnson and Terkeltaub 2003; Scharstuhl et al. 2003; Glasson et al. 2004, 2005; Ueblicher et al. 2004; Ford-Hutchinson et al. 2005; Wadhwa et al. 2005; Xu et al. 2005; Zaka and Williams 2005; Zhang et al. 2005).

It should be noted that differences exist in the pathomechanisms of cartilage destruction between rheumatoid arthritis (RA) and OA. The destruction occurring in RA is closely linked to the inflammatory process, synovial tissue proliferation and transgression across the cartilage surface, degrading cartilage proteoglycans and collagens simultaneously. In OA inflammation is only an intermitted event, not instrumental in the degenerative cartilage destruction, in which proteoglycan degradation is the early event, and collagen loss occurs at a distinctly later stage. Therefore, animal models with a predominant inflammatory component as the air pouch model or other arthritis models, even those focusing on cartilage destruction, are not discussed in this chapter (see Sect. H.3). They are described in detail by Greenwald and Diamond (1988) and recently reviewed by Greenwald (1991, 1993).

PURPOSE AND RATIONALE

As a multitude of different events can lead to OA, equally different techniques have been used to initiate osteoarthritic conditions in animal models: Surgi-

cal methods are used to either stiffen the joint in a defined position (Palmoski, Brandt 1982; Konttinen et al. 1990; Meyer-Carrive and Ghosh 1992; Torelli et al. 2005) or inflict joint instability by partial meniscectomy or anterior crucial ligament (ACL) dissection. They are performed mainly in dogs, rabbits (Colombo et al. 1983; Moskowitz et al. 1973, 1979), and guinea pigs (Schwartz 1985; Meacock et al. 1990). Chemical modifications like intraarticular injections of iodoacetate, cytokines like IL-1, or enzymes like chymopapain or stromelysin, are carried out mainly in rabbits (Williams et al. 1992; Reglin et al. 1989), in chicken (Kalbhen 1983, 1987), and in rats (Combe et al. 2004). Mechanical forces are applied on bent or opened joints like impulse loading on sheep (Lindenhayn et al. 1984) or rabbit (Farkas et al. 1987; Mazière et al. 1984) knees, resulting in trauma models of OA. Spontaneously occurring OA is described in horses (Todhunter and Lust 1992; Haakenstad 1969), some breeds of dogs (Lust et al. 1985), rhesus macaques (Pritzker et al. 1989), guinea pigs (Bendele, Hulman 1988) and several strains of mice. For pharmacological purposes only guinea pigs and STR/IN (Walton 1965; Raiss et al. 1992), STR/ORT (Dunham et al. 1989), and C57 black (Pataki et al. 1990) mice have been adapted. In all models mentioned (except horses), the relevant joint is the knee.

Since there exist recent extensive and critical reviews of OA models (Burton-Wurster et al. 1993; Pritzker 1994; Moskowitz 1990, 1992; Adams and Billingham 1982; Oegema et al. 1999; Bendele 2001, 2002; Bonnet and Walsh 2005), also with respect to reversibility (Pita et al. 1986), with special emphasis on drug testing (Hess & Herman 1986; Hinz and Brune 2004; Wieland et al. 2005), and in perspective to cartilage research and markers (Malemud 1993; Carney 1991), only some representative models of each category are described here.

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These authors achieved the ACL transection originally through a lateral stab incision, whereas others also performed the ligament transection after opening the joint. Both versions lead to similar morphological and biochemical changes, and are described and illustrated in detail by Adams and Pelletier (1988).

PROCEDURE

Animals

Mongrels, beagles, greyhounds, and foxhounds are reported to be suitable, provided purebred strains are used. In general, younger animals display more repair phenomena, whereas older ones seem to show more rapid degeneration. As the epiphyses fuse at the age of 13 months, and as pronounced changes require up to 4 months to fully develop, careful planning ahead is essential.

Operation

The dogs are initially anesthetized with 30 mg/kg sodium pentobarbital i.v., followed by a continuous inhalation of 1% halothane with 1 l/min N₂O and 2 l/min O₂. After shaving and sterilizing the knee joint externally, it is fixed in a bent position at 90°, and a scalpel blade is inserted medially deep into the joint space diagonally posterior to the ACL, and parallel to the lateral border of the patellar ligament. By rotation of the blade, the ACL is then dissected, the blade withdrawn, and the wound closed. In the contralateral knee, a sham operation is performed to inflict similar disturbance to the joint tissue, but without harming the ACL. The ACL dissection results after 8 to 12 weeks, in contrast to the sham-operated contralateral knee, in fibrillation and erosion of the cartilage, more pronounced on the tibial plateau than on the femoral condyles. Also observed histologically is a loss of metachromatic staining, and a fissured surface with cell clones appears. A marked osteophytosis and subchondral sclerosis has also developed at that time. Proteoglycan content and overall cartilage thickness, however, seems to remain stable (Pelletier and Martel-Pelletier 1985) or even increase not only for several months (McDevitt et al. 1977; Vignon et al. 1983; Brandt and Adams 1989), but for up to 3 years after transection, as Brandt, Myers et al. (1991) could show. At later stages, however, severe cartilage thinning and loss is recorded (Brandt, Braunstein et al. 1991).

EVALUATION

Macroscopic inspection of cartilage and osteophytes are recorded. Histological grading based on the Mankin score has been reported to be modified by drug

1.1.2.2

Canine Anterior Cruciate Ligament (ACL) Transection Model

PURPOSE AND RATIONALE

Similar to human ACL ruptures, ACL transection in the dog knee and the subsequent joint instability results in progressive cartilage erosion, fibrillation, and formation of osteophytes. This elaborate model is well characterized, and regarded of high predictive value. It is mostly known as the “Pond-Nuki dog model”.

treatment over 7 weeks (Abatangelo et al. 1989; Schiavinato et al. 1989). As levels and activity of neutral matrix metalloproteases are elevated in cartilage and synovium (Pelletier, Martel-Pelletier 1985), they might be additional parameters of interest to profile the test compounds.

CRITICAL ASSESSMENT OF THE TEST

In this instability model, a polysulfated glycosaminoglycan preparation (Arteparon), as well as intra-articular injections of a hyaluronic acid preparation induced some morphological and biochemical changes, whereas low-dose prednisone had no effect. As there seems to prevail an anabolic response to the instability in the articular cartilage for quite a long time, the selected time points and parameters to assess disease progression and therapeutic success require careful consideration.

MODIFICATIONS OF THE TEST

Caron et al. (1996) and Pelletier et al. (1997) investigated the *in vivo* effect of the recombinant human interleukin-1 receptor antagonist on the development of lesions in the anterior cruciate ligament transection model in dogs.

Wenz et al. (1998) used the POND-Nuki model in dogs to evaluate the effectiveness of intra-articular application of hyaluronic acid on early forms of femoropatellar arthrosis. Transection of the anterior cruciate ligament in dogs was also used in studies by Myers et al. (1999), Boileau et al. (2002, 2005), Smith et al. (1999, 2002), Behets et al. (2004), and Matyas et al. (2004).

Pelletier et al. (2005) studied the effects of licochalcone, a 5-lipoxygenase/cyclooxygenase inhibitor, in the experimental anterior cruciate ligament dog model of osteoarthritis.

ACL transection can be performed also in an arthroscopy operation as described by Adams and Pelletier (1988), and an instability can be achieved equally by meniscectomy (Hannan et al. 1987).

The **rabbit** is the main other species used for instability models, as described in detail for partial medial meniscectomy by DiPasquale et al. (1988), and for partial lateral meniscectomy in connection with ligament transection by Colombo (1988).

Obara et al. (1993) induced osteoarthritis by surgical dissection of the anterior cruciate ligament in rabbits and investigated fluorescence distribution after intra-articular administration of fluorescein-labeled sodium hyaluronate.

Anterior cruciate ligament transection in rabbits was used in several studies (Amiel et al. 2003; Kawano et al. 2003; Doschak et al. 2004; Zhang et al. 2004; Diaz-Gallego et al. 2005; Tiralocche et al. 2005).

Kobayashi et al. (2005) studied a vitamin B1 derivative, and its ability to enhance the chondroprotective effects of glucosamine hydrochloride and chondroitin sulfate in osteoarthritis created by partial medial meniscectomy of the knee joint in rabbits.

A partial medial meniscectomy with ligament transection is described for the **guinea pig** by Bendele (1987) and Schwartz (1988). This method has been used by Bendele et al. (1999) and by Sabatini et al. (2005).

Layton et al. (1987) produced biomechanical stress-induced hip osteoarthritis in guinea pigs by extra-articular myectomy and tendotomy.

The Hartley guinea pig strain spontaneously develops knee osteoarthritis whose manifestations are markedly similar to those of spontaneous knee osteoarthritis in humans (Bendele et al. 1989; Kraus et al. 2004).

Janusz et al. (2002) described a model of osteoarthritis in **rats** induced by surgically transecting the medial collateral ligament and meniscus and evaluated the effectiveness of a matrix metalloproteinase inhibitor in this model. Using this model, Moore et al. (2005) found that fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair.

Machner et al. (1999) investigated the influence of an altered sensible joint innervation on the development of knee osteoarthritis in Wistar rats. Partial sensible joint denervation was performed by injection of 100 µg capsaicin into a sponge placed on the sensible nerve trunk. Half of the rats underwent strenuous running exercises (20 km in a running wheel by intracranial self-stimulation). Under these conditions, severe osteoarthritis changes were observed, which were only mild without exercise.

Hayamai et al. (2004) studied cartilage degeneration and osteophyte formation in the anterior cruciate ligament transection model in rats.

Wancket et al. (2005) used the medial meniscectomy model in Lewis rats to study the anatomical localization of cartilage degradation markers.

Ghosh et al. (1993), Appleyard et al. (1999), and Burkhart et al. (2001) used an osteoarthritis model induced by lateral meniscectomy in **sheep**.

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I.1.2.3

Chymopapain-Induced Cartilage Degeneration in the Rabbit

PURPOSE AND RATIONALE

Intraarticular injection of chymopapain into the rabbit knee joint results in cartilage degradation with rapid loss of proteoglycans. A transient inflammation shortly after injection normally subsides after 1–2 days. Severity and reversibility of the cartilage damage can be altered using different protocols (Williams et al. 1992).

PROCEDURE

Most authors use New Zealand White rabbits at the age of 2 to 4 months. Male Chinchilla and Chinchilla Bastard rabbits are preferred, as they display a thicker cartilage than the New Zealand strain. The animals with an initial body weight of ca 2.5 kg are anesthetized with a continuous inhalation of 4% halothane – 3 l/min N₂O – 1.8 l/min O₂. Both knee joints are carefully shaved and moisturized with 70% alcohol. With a sterile syringe a volume of 0.1 ml of the following solution is injected laterally into the joint space: eighteen mg chymopapain (Sigma) are dissolved in 1 ml 0.9% NaCl, and 50 mg l-cysteine-HCl is added for activation. The preparation is then passed under sterile conditions through a 0.22 µm Millipore filter, and tested for activity prior to each operation. (Potential direct interference of the therapeutic agent to be tested with the chymopapain can also be addressed at that point). One injection of 1.8 mg chymopapain in 0.1 ml per joint usually results in a proteoglycan loss/cartilage

dry weight of ca 40% after 10 to 12 days. Two to 4 animals per experiment receive chymopapain in 0.9% NaCl in one joint, and 0.9% NaCl into the contralateral knee to assess the proteoglycan loss caused by the enzyme. Care is taken with all injections to apply the same volume to both joints, as the contralateral serves as internal control. If the drug is given orally, only one knee receives chymopapain treatment, whereas if the drug is applied intraarticularly into one knee (1 to 5 injections of 0.1 ml volume each, vehicle into contralateral joint, in a period of 5 to 10 days), both knees are treated with chymopapain. The animals are sacrificed after 10 to 14 days, and from defined regions of weight-bearing areas in the joint full thickness samples are obtained for histology and assessment of proteoglycan content.

Histology is performed on full thickness sections of the articular cartilage, fixed with 3% formalin, embedded in paraffin, and stained routinely with safranin-O/fast green, or with toluidin-blue (Romeis 1989).

The proteoglycan content is determined with the dimethyl-methylen blue (DMB) method modified after Farndale et al. (1986) and Chandrasekhar et al. (1987). The wet weight of the cartilage samples is determined immediately after preparation, and their dry weight is recorded after 3 day drying at 60°C. Samples are then soaked overnight in 1 ml buffer (containing 20 mM disodiumhydrogenphosphate, 1 mM EDTA, and 2 mM dithiothreitol in 500 ml aqua bidest at pH 6.8). Ten μ l of papain, suspended in 0.05 M sodiumacetate (Sigma 9001-73-4), are then added, and incubated at 60°C for 6 h. Ten μ l of standard (shark chondroitinsulfate C (Sigma C4384) in a concentration range from 10–200 μ g/ml buffer) and of each probe are then transferred into a 96 well microtiter-plate. Two hundred μ l of DMB is added, and, after 1 min, the extinction recorded photometrically at 690/540 nm. The DMB solution is prepared by dissolving 16 mg DMB (Serva 20335) in 2 ml methanol, adding 400 ml aqua bidest, 9.6 ml 1N HCl-solution, 3.04 g glycine, and 2.36 g NaCl, stirred and warmed until dissolved, and then diluted to a final volume of 1000 ml with aqua dest (pH 3.0). The results are recorded as μ g chondroitinsulfate equivalents/mg cartilage dry weight.

EVALUATION

The histological appearance of the articular cartilage is assessed with a modified Mankin score, emphasizing more earlier changes, and separating degradative aspects, (e.g. loss of safranin staining), from repair aspects, (e.g. the occurrence of pericellular staining). The proteoglycan content is expressed as % change

to the contralateral knee, or, comparing groups, to chymopapain-treated or untreated control groups. To relate the proteoglycan content to cartilage dry weight has been found to be more reliable than choosing wet weight as baseline, as the cartilage water content is known to change in this model according to matrix composition.

CRITICAL ASSESSMENT OF THE TEST

In this model, hyaluronic acid preparations and some MMP inhibitors can be detected, NSAIDs do not change the parameters tested. The protocol can be modified to emphasize repair phenomena, or to assure a more severe course of cartilage loss (Williams et al. 1993).

MODIFICATIONS OF THE MODEL

Muehleman et al. (2002) investigated bone remodeling inhibition by a biphosphonate in the rabbit model of cartilage matrix damage by chymopapain injection.

To avoid interaction of a drug with the enzymatic nature of the chymopapain, other degradation mediators can be applied instead, as e.g. fibronectin fragments (Williams et al. 1988).

To ensure continuous synovial levels of a given drug, an osmotic minipump containing the drug can be implanted into the femoral muscles with a prolonged fine tube to insert into the patellar groove (Karbowski et al. in prep). This is an elaborate procedure in which a Ketavet/Rampun anesthesia is needed, and postoperative plastic collars to prevent the rabbits to interfere with the operated region for the first three days. This results in highly consistent data compared to repeated injections.

Kikuchi et al. (1998) induced experimental osteoarthritis in mature rabbits by intra-articular injection of collagenase.

Pomonis et al. (2005) developed a **rat model of osteoarthritis pain**. Male Sprague Dawley rats were anesthetized with isoflurane and received a single injection of iodoacetate (0.3, 1, or 3 mg), papain (0.5, 1, 2, or 3%) or saline in a 50 μ l volume into the left knee joint using a 27-gauge needle inserted through the patella tendon. Assessment of pain-related behaviors was performed using a hind limb weight-bearing apparatus (Linton Incapacitance Tester Stoelting, Wood Dale, Ill., USA). Animals treated with intra-articular iodoacetate were evaluated for alterations in weight bearing after the acute and chronic administration of various drugs.

Osteoarthritic lesions in the knee joints of male C57b110 mice were induced by a single intra-articular

injection of bacterial collagenase (Van der Kraan et al. 1990; Van den Berg et al. 1993).

Van der Kraan et al. (1989) and Scharstuhl et al. (2002) induced arthritis in mice by a single intra-articular injection of papain in their knee joints.

Van Osch et al. (1995) developed a device to measure laxity of knee joints in mice. Reproducible, non-linear S-shaped load displacement curves were determined from knee joints of normal mice. Parameters of anterior-posterior translation, varus-valgus rotation, and compliance were calculated from the curves. Laxity was markedly increased in animals with osteoarthritis induced by intra-articular injection of collagenase.

Saez-Llorens et al. (1991) and Cohen et al. (2004) described a model of **septic arthrosis** in rabbits.

Four-month-old female New Zealand rabbits were anesthetized with an intramuscular injection of 60 mg/kg ketamine and 6 mg/kg xylazine. The skin overlying the knees was shaved and cleansed with povidone-iodine and 70% ethanol. The right knee was injected with 10^5 – 10^6 colony forming units of *Staphylococcus aureus*, while the left knees were injected with sterile saline. Following injection, all rabbits were allowed normal cage activity. The rabbits were left either untreated, or were treated with antibiotic (40 mg/kg ceftriaxone) alone or additionally with different doses of test drug. Animals were anesthetized at 1, 4, and 7 days following initiation of treatment. Prior to aspiration of synovial fluid, the knee joint was inspected for evidence of effusion and range of motion testing. Following an arthrotomy, the gross appearance of the joint was examined for joint degradation and the fluid grossly inspected for purulence. Prior to sacrifice, an arterial blood sample was plated on agar plates, incubated for 48 h at 37°C, and analyzed for colony growth. In the synovial fluid the number of white blood cells was determined and bacterial growth examined in serial dilutions. Histology was performed in synovial tissue from the pre-patellar fat pad and the medial femoral condyle.

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I.1.2.4

Spontaneous OA Model in STR/1N Mice

PURPOSE AND RATIONALE

This spontaneous model has the advantage of a gradually developing OA starting with cartilage surface ero-

sion and fibrillation to osteophyte formation, subchondral bone remodeling, and finally eburnation, in a moderate time frame. All these steps, with only intermittent inflammatory flares, are similar to human pathology, and develop without any interference. The disadvantages are, however, the restriction to systemic drug application, due to the relative small animal size, and the variance in onset and severity of the disease among the animals.

PROCEDURE

The STR/IN mouse strain, characterized extensively by Walton (1977–1979) can be obtained from the National Institute of Health, Bethesda MA, USA. It is not identical to the STR/ORT strain as described by Benjamin et al. (1995), since it displays, in contrast to the ORT, no calcification in ligaments or tendons. Male mice develop OA earlier and more consistently than females, and, without dietary restriction, develop additionally obesity.

Beginning at the age of 10 weeks, male mice are trained to walk on a slowly rotating cylinder (or treadmill, manufactured by Ugo Basili, Italy) recording the mean walking time of each mouse. Mice with a moderate activity (neither dropping off too soon, nor staying on for hours) are then selected to enter the experiment. In groups of 8 to 10 animals, the drug is applied systemically for 8 weeks, and the mobility of each animal is recorded once or twice a week on the rotating cylinder. The body weight is recorded regularly as well. At the end of the experiment, the animals are sacrificed, both knee joints dissected, fixed, decalcified, and embedded in defined orientation for histology.

EVALUATION

The mean walking time decreases with age and disease progression, and is recorded in a time-dependent graph over the treatment period. The medial tibial plateau of the knee joints exhibits the most pronounced cartilage fibrillation and loss, and is, therefore, selected for histological evaluation, based on a modified Mankin grade.

CRITICAL ASSESSMENT OF THE TEST

Since this model is best suitable for oral drug application, experience with disease-modifying drugs for osteoarthritis is limited. Drugs with anti-inflammatory properties increase walking time, but do not alter the morphological aspects of cartilage degradation in the joint. Analgesic drugs have no effect on either.

MODIFICATIONS OF THE TEST

Rudolphi et al. (2003) studied an inhibitor of interleukin-1 β converting enzyme in STR/IN mice and in a collagenase-induced osteoarthritis model in Balb/c mice. Weakening of knee joint ligaments was achieved by intra-articular injection of highly purified bacterial collagenase leading to increased joint laxity which resulted in osteoarthritic lesions preferentially in the medial compartments of the injected knee joint (Van der Kraan et al. 1990).

Several authors (Nakamura 1990; Pataki and Witzemann 1990; Wilhelmi and Meyer 1983) have used the C57black mouse which, however, is reported to vary considerably in the incidence and severity of the disease, and also to develop the osteoarthritis only at older ages.

The STR/ORT strain differs in its calcification of fibrous cartilage (Benjamin et al. 1995), and seems to change the orientation of the proteoglycans in the hyaline cartilage (Dunham et al. 1989).

Gaffen et al. (1997) found elevated aggrecan mRNA in STR/ort mice. Brewster et al. (1998) tested an orally active collagenase inhibitor in STR/ORT mice. Microfocal X-ray-generated images of the hind limbs as well as histologic sections of the knees were scored for degradative changes and drug effects. Chambers et al. (2001) reported that matrix metalloproteinases and aggrecanases cleave aggrecan in different zones of normal cartilage but co-localize in the development of osteoarthritis lesions on STR/ort mice. Mason et al. (2001) reviewed the use of the STR/ort mouse as a model of osteoarthritis. Flannelly et al. (2002) studied the temporal expression of matrix metalloproteinases and tissue inhibitors in the murine STR/ort model of osteoarthritis. Price et al. (2002) compared collagenase-cleaved articular cartilage collagen in mice in the naturally occurring STR/ort model of osteoarthritis and in collagen-induced arthritis.

Glant et al. (1998) reported progressive polyarthritis induced in BALB/c mice by aggrecan from normal and osteoarthritic human cartilage.

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I.1.2.5

Transgenic Mice as Models of Osteoarthritis

PURPOSE AND RATIONALE

Osteoarthritis comprises a group of diseases characterized by gradual degeneration of articular cartilage and a number of associated processes within the joint. Consequently, no single animal model based on one genetic alteration is likely to fulfill all the criteria of human osteoarthritis (Helminen et al. 2002; Säämänen and Vuorio 2004). Transgenic mice with **collagen mutations** include different types of collagen.

Several lines of transgenic mice have been produced harboring mutations in the type II collagen gene. These include a line expressing a human *COL2A1* transgene with a large internal deletion (Vandenberg et al. 1991), and another expressing a murine *Col2a1* transgene with a Gly58Cys substitution in the helical domain (Garofalo et al. 1991). An osteoarthritis phenotype can also be produced by increasing the expression rate of the normal *Col2a1* gene, which leads to disruption of the regulation of type II collagen fibril assembly (Garofalo et al. 1993). The transgenic mouse line Dell harbors six copies of a *Col2a1* transgene with a 150-bp deletion of exon 7 and introns 7, removing sequences coding for the 15 amino acids at the amino-terminal end of the triple helical domain (Metsäranta et al. 1992). Homozygous Dell mice die at birth due to respiratory distress. Heterozygous Dell mice develop early-onset human osteoarthritis-like lesions, usually confined to the knee joint (Säämänen et al. 2000).

Mice transgenic for type IX collagen mutations carrying a central in-frame deletion mutation that codes for truncated $\alpha 1$ (IX) chains develop mild chondrodysplasia and progressive osteoarthritis with ocular involvement (Nakata et al. 1993). Fässler et al. (1994) reported non-inflammatory degenerative joint disease in mice lacking $\alpha 1$ (IX) collagen.

Xu et al. (2003) demonstrated osteoarthritis-like changes and decreased mechanical function of articular cartilage in the joints of mice with the chondrodysplasia gene (*cho*). A mutation in the gene encoding the $\alpha 1$ chain of type XI collagen (*Col11a1*) was identified as the genetic cause of chondrodysplasia in mice.

Several transgenic models of osteoarthritis with mutations in non-collagenous molecules were described. Ameye et al. (2002) reported that abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. Ameye and Young (2002) recommended mice deficient in small leucine-rich proteoglycans as novel *in vivo* models of osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases.

Zemmyo et al. (2003) described accelerated, aging-dependent development of osteoarthritis in $\alpha 1$ integrin-deficient mice. Morko et al. (2004) reported upregulation of cathepsin K expression in articular chondrocytes in a transgenic mouse model of osteoarthritis.

Involvement of **matrix metalloproteinases** has been demonstrated in the degradation of cartilage and the development of human osteoarthritis lesions. Holmbeck et al. (1999) reported that MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Neuhold et al. (2001) found that postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. These mice develop cartilage lesions with histological similarities to human osteoarthritis. Salminen et al. (2002) reported differential expression patterns of matrix metalloproteinases and their inhibitors during development of osteoarthritis in the transgenic D ℓ 1 mouse model. Glasson et al. (2005) reported that deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis.

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I.2 Methods for Testing Immunological Factors

I.2.1 In Vitro Methods

I.2.1.1 Inhibition Of Histamine Release from Mast Cells

PURPOSE AND RATIONALE

Hypersensitivity reactions can be elicited by various factors: either immunologically induced, i. e. allergic reactions to natural or synthetic compounds mediated by IgE, or non-immunologically induced, i. e. activation of mediator release from cells through direct contact, without the induction of, or the mediation through immune responses. Mediators responsible for hypersensitivity reactions are released from mast cells. An important preformed mediator of allergic reactions found in these cells is histamine. Specific allergens or the calcium ionophore 48/80 induce release of histamine from mast cells. The histamine concentration can be determined with the *o*-phthalaldehyde reaction.

PROCEDURE

Preparation of Mast Cell Suspension

Wistar rats are decapitated and exsanguinated. Fifty ml of Hank's balanced salt solution (HBSS) are injected into the peritoneal cavity and following massage of the body, the abdominal wall is opened. The fluid containing peritoneal cells is collected in a centrifuge tube and centrifuged at 2000 rpm. The cells are resuspended in HBSS. Then the cell suspension is brought to a final concentration of 10^5 mast cells/100 μ l.

Test Compound Administration and Induction of Histamine Release

1 ml test drug (concentration range between 10^{-4} and 10^{-8} Mol) is added to the mast cell suspension (10^5 cells/100 ml) and the mixture is incubated at 37°C for 15 min. The cells are made up to a volume of 3 ml with HBSS, an equal volume of calcium-ionophore (10^{-6} g/ml), compound 48/80 or specific allergen is added. The suspension is incubated at 37°C for 30 min followed by centrifugation at 2500 rpm.

The Following Control Solutions are Needed

- *Spontaneous histamine release*: contains only mast cells and solutions used to determine baseline.
- *Histamine release*: contains mast cells and solutions and calcium-ionophore (10^{-6} g/ml).

- *Test compound control*: contains solutions and test compound to test the compound for native fluorescence.
- *Solution control*: contains only solutions used in the test to determine baseline.

Extraction of Histamine

One ml of the top layer is transferred to a tube containing 300 mg NaCl and 1.25 ml butanol. The sample is alkalinized to extract the histamine into butanol by adding 1 ml 3 N NaOH. Following mechanical shaking, the sample is centrifuged for 5 min. One ml of the top layer (butanol) is pipetted into a 5 ml tube containing 2 ml of n-heptane and 0.4 ml of 0.12 N HCl. The tube is mixed by inverting it several times. Following separation into aqueous and organic phases, 0.5 ml of the aqueous phase is transferred to another tube.

Induction of *o*-Phthalaldehyde Complexing Reaction

To each sample 100 μ l 1 N NaOH is added under constant stirring immediately followed by administration of 100 μ l 0.2% *o*-phthalaldehyde solution. After 2 min, the *o*-phthalaldehyde complexing reaction is stopped by addition of 50 μ l 3 N HCl.

Determination of Histamine Release

The total sample is transferred to an autosampler vial and the histamine concentration is determined by a fluorescence detector (using excitation and emission wave lengths of 350 and 450 nm respectively).

EVALUATION

Percent histamine release (hist. rel.) can be expressed by the following formula:

$$\frac{\text{sample hist. rel.} - \text{spontaneous hist. rel.}}{100\% \text{ hist. rel.} - \text{spontaneous hist. rel.}} \times 100$$

The statistical evaluation is carried out using the Student's *t*-test (comparison of 100% control to experimental group)

CRITICAL ASSESSMENT OF THE METHOD

Disodium cromoglycate has been reported to inhibit the release of histamine and the degranulation of rat mast cells (Orr and Cox 1969; Orr et al. 1971; Johnson and Bach 1975; Church and Young 1983). However, this effect of disodium cromoglycate and its analogues does not parallel the clinical efficacy (Kay et al. 1987).

MODIFICATIONS OF THE METHOD

Johnston et al. (1978) studied the increased superoxide anion production by immunologically activated and chemically elicited macrophages.

Flint et al. (1985) found a significant inhibition of histamine release by disodium cromoglycate in human mast cells recovered by bronchoalveolar lavage.

Ali et al. (1985) investigated the histamine release from rat peritoneal mast cells, human basophil and neutrophil leukocytes, mast cells from mesentery lung and heart of rats and guinea pigs by the skin irritating constituents thapsigargin and thapsigarginin from the resin of the umbelliferous plant *Thapsia gargania*.

Eady (1986) studied the reactivity of mast cells in bronchoalveolar lavage fluid of macaques repeatedly infected with *Ascaris suum*.

Wells et al. (1986) compared release of histamine, LTC₄, and PGD₂ from primate bronchoalveolar mast cells with that of rat peritoneal mast cells.

The release of β -hexosaminidase from mouse or rat bone marrow derived mast cells and from rat peritoneal mast cells was studied by Broide et al. (1986).

Peretti et al. (1990) recommended flow cytometry to investigate mast cell degranulation. Peptides, including substance P and bradykinin analogs release histamine from human skin mast cells (Lawrence et al. 1989).

Williams et al. (1991) studied the vancomycin-induced release of histamine from rat peritoneal mast cells and a rat basophil cell line (RBL-1).

A sensitive colorimetric assay for the release of tryptase from human lung mast cells *in vitro* has been described by Lavens et al. (1993).

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1.2.1.2**Mitogen Induced Lymphocyte Proliferation****PURPOSE AND RATIONALE**

Cultured lymphocytes can be stimulated to a proliferative response and to DNA synthesis by various mitogens. Measurement of DNA synthesis can be accomplished by pulse-labeling the culture with tritiated thymidine (³H-thymidine), a nucleoside which is incorporated into the newly synthesized DNA. Immunomodulating properties can be detected either by pre-

treatment of the animals *in vivo* or by adding the test drug to the cultured lymphocytes.

PROCEDURE

Mice of NMRI strain weighing 18–20 g or rats of Lewis strain weighing 180–200 g are used.

Materials

Sheep red blood cell (SRBC) specific antigen and/or the following mitogens:

- Lipopolysaccharide 10–0.1 µg/ml
- Dextran sulfate 30–7.5 µg/ml
- Phytohaemagglutinin 0.5–0.12 % stock solution
- Concanavallin A 0.5–0.12 µg/ml
- As standards levamisole, cyclosporine A, prednisolone or leflunomide are used.

Ex Vivo

Animals receive the test compound once a day for 5 days. Thereafter, they are sacrificed, spleens are removed and a single cell suspension of 5×10^6 cells/ml is prepared. Mitogens are titrated (4 replicates/group) in 0.1 ml/well and 0.1 ml of the cell suspension is added. Plates are incubated at 37°C in 5% CO₂ in air for 48–60 h and for another 8 h after addition of 0.25 µC ³H-thymidine per well. Cells are harvested on glass fiber filters and after drying the degree of radioactivity is determined using a β-counter.

In Vitro

Animals are sacrificed and their spleens removed. A single cell suspension of 10⁷ cells/ml is prepared and 0.05 ml placed in each microtiter well (4 replicates/group). Then the test compound (4 times concentrated) is added in 0.05 ml. At last 0.1 ml of the double concentrated mitogen is added. Plates are incubated and processed as described above.

EVALUATION

Stimulation index = proliferation ratio according to positive control, either with or without mean spleen weight. Statistical evaluation is carried out using the Student's *t*-test (comparison of positive and/or negative control to experimental group).

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I.2.1.3

Inhibition of T Cell Proliferation

PURPOSE AND RATIONALE

Activation and/or proliferation of clonal populations of T cells are critical for the initiation of an antigen-specific immune response. Thus, inhibition of T cell activation provides a potent means for suppressing specific immune response. A number of immunosuppressive agents exhibit the ability to suppress T cell activation.

PROCEDURE

Purification of Peripheral Blood Leukocytes and T Cells

Peripheral blood leukocytes from normal donors are separated on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Leukocyte suspensions are washed in HBSS and are resuspended in RPMI 1664 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. Leukocyte suspensions are resuspended in RPMI 1664 containing 10% heat-inactivated pooled human serum. Highly enriched T cells are obtained by passing leukocytes through a nylon wool column to remove macrophages and B cells and then depleted of NK and monocytes with anti-Leu 11 b (Becton Dickinson, Mountain View, CA) plus complement (Pel-Freez, Brown Deer, WI). These highly enriched T cells are approximately 95% CD3+ cells, the remaining cells being B lymphocytes.

Mixed Lymphocyte Reaction

Peripheral blood leukocytes are incubated at 2×10^5 /well with equal numbers of gamma-irradiated (3000 rads) allogenic peripheral blood leukocytes and various concentrations of test compounds. Assays are performed in triplicate in 96-well, U-bottom plates. After 6 days of coculture, the cells are pulsed for 6 h with 1 µC of [³H]thymidine per well. [³H]Thymidine incorporation is then measured by scintillation

counting. Data are presented as:

$$\% \text{ inhibition} = \frac{\text{CPM}_{\text{expt}} - \text{CPM}_{\text{bckgrd}}}{\text{CPM}_{\text{ctrl}} - \text{CPM}_{\text{bckgrd}}} \times 100$$

where CPM_{expt} is mean counts per min of experimental cultures, $\text{CPM}_{\text{bckgrd}}$ is mean counts per min of background well, unstimulated cultures, and CPM_{ctrl} is mean counts per min of uninhibited, stimulated cultures.

Lymphocyte Stimulation and Proliferation

Peripheral blood leukocytes and isolated T cells are cultured with anti-CD3 (5 ng/ml) plus PMA (5 ng/ml), anti-CD28 (1:5000 dilution) plus PMA (5 ng/ml), or 100 U/ml rhuIL-2 in RPMI 1644 containing 10% fetal bovine serum. Peripheral blood leukocytes or T cells are cultured at 2×10^5 cell per well in a total volume of 200 μ l/well. Assays are performed in quadruplicate in 96-well, U-bottom plates. [^3H]Thymidine (1 μ C) is added to each well after 48 h of coculture and after a 20 h pulse of [^3H]thymidine, the cells are harvested and the amount of [^3H]thymidine uptake is quantitated on a scintillation counter.

ELISA Assays

Supernatants/well (100 ml) are harvested 24 h after initiation of cultures of peripheral blood leukocytes or T cells stimulated with anti-CD3 or anti-CD28 plus PMA. IL-2 in the coculture supernatant is quantitated using a commercially available IL-2 ELISA kit. All experiments are performed in duplicate.

IL-2R Assays

The expression of IL-2R on T cells stimulated for 48 h with anti-CD3 or anti-CD28 plus PMA is determined using FITC-conjugated anti-CD25 mABs (Becton Dickinson, Mountain View, CA). T cells are washed in HBSS and then stained with phycoerythrin-conjugated anti-CD3 mAB and fluorescein-conjugated anti-CD25 mAB. The percent of cells coexpressing CD3+ and CD25+ are determined from 2000 cells using an EPICS C flow cytometer (Coulter, Hialeah, FL).

EVALUATION

Dose response curves of inhibition of one-way mixed lymphocyte reaction and of IL-2 in the supernatant after stimulation with anti-CD3 or anti-CD28 are established.

MODIFICATIONS OF THE METHOD

Zielinski et al. (1993, 1994) studied the influence of leflunomide on expression of lymphocyte activation

expression markers (IL-2 and transferrin receptors) as well as on cell cycle and on IL-2 receptor gene expression.

Calcineurin was found to be a key signaling enzyme in T lymphocyte activation and the target of immunosuppressive drugs (Clipstone and Crabtree 1993).

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1.2.1.4

Chemiluminescence in Macrophages

PURPOSE AND RATIONALE

The stimulation of macrophages by antigen, complement, phorbol-esters, ect. leads to elaboration of O_2^- and other oxygen metabolites. Superoxide ion (O_2^-) and other highly reactive oxygen metabolites (radicals) form the basis for an efficient microbicidal system *in vivo*. Yet, when these radicals are released in response to self-antigens, tissue damage is often the result. Inhibition of this process can be regarded as a measure for immun-modulating effects of compounds. The oxygen metabolites can produce light-emitting reactions (chemiluminescence), which is measurable if amplified with suitable agents, such as the cyclic hydrazide luminol.

PROCEDURE

NMRI mice weighing 30 g or Sprague-Dawley rats weighing 250–300 g of either sex are used.

Positive Control

1. Sensitized mice, receiving vehicle
2. Mice, developing an autoimmune disease, receiving vehicle
3. Rats, developing adjuvant arthritis, receiving vehicle

Negative Control

1. Mice not sensitized, receiving vehicle
2. Mice, not developing an autoimmune disease, receiving vehicle
3. Rats without adjuvant arthritis.

Materials

- 5×10^8 SRBC (sheep red blood cells)/0.5 ml 0.9% NaCl solution (for sensitization)
- *Phorbol ester*: Stock solution of 1 mg/ml phorbolmyristenacetate. This stock solution is diluted with Hank's balanced salt solution to a final concentration of 3.5 μ M (working solution). For the induction of chemiluminescence, the working solution is diluted in the test tube 1:4, resulting in a final phorbol ester concentration of 0.875 μ M.
- Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) final concentration 25 μ g/ml

Ex Vivo Experiment

Groups of 6 animals are treated for 6 days orally or subcutaneously with test compound or the standard (prednisolone acetate or leflunomide). They are decapitated and exsanguinated. Macrophages are obtained by flushing the peritoneal cavity with 10 ml saline, containing 250 IU heparin. The cells are pooled, washed several times and suspended again at a final concentration of $2 \times 10^6/200 \mu$ l.

For measurement in the luminometer the following mixture is prepared:

200 μ l macrophages (2×10^6)

100 μ l luminol solution (100 μ g/ml)

100 μ l phorbolmyristenacetate solution (3.5 μ M)

Each sample is mixed thoroughly without the phorbolmyristenacetate solution, put into the luminometer and counted at 2 min intervals for 10 s. The addition of the phorbol ester induces the reaction.

In Vitro Experiment

To 100 μ l of macrophage suspension (2×10^6 cells) are 100 μ l of the solution of the test compound added and incubated for 15 min at 37°C.

Then, 100 μ l of luminol solution (100 μ g/ml) and 100 μ l of the 3.5 μ M phorbol ester solution are added and the luminescence measured in the luminometer.

EVALUATION

The time of maximal counts for the positive control is recorded. For all groups the ratio of counts per 10 s is determined at that time, compared to the positive control counts per 10 s and the percent change is calculated. For statistical evaluation the experimental group is compared with the positive control group using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Bird and Giroud (1985) described a technique of polymorphonuclear leukocyte chemiluminescence as a means to detect compounds with anti-inflammatory activity. Inflammatory polymorphonuclear leukocytes were obtained by injecting rats intrapleurally with 1 ml of a 1% solution of calcium pyrophosphate and collection of the pleural exudate 4 h later. Chemoluminescence responses were measured using a Packard Pico-lite chemoluminometer and opsonized zymosan as the stimulus.

Seeds et al. (1985) found an independent stimulation of membrane potential changes and the oxidative metabolic burst in polymorphonuclear leukocytes.

A microtechnique for studying chemiluminescence response of phagocytes using whole blood was described by Selvaraj et al. (1982).

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1.2.1.5

PFC (Plaque Forming Colony) Test In Vitro

PURPOSE AND RATIONALE

Identification of antibody producing cells is based on the ability of the secreted IgM antibody to fix complement and thereby lyse the indicator erythrocytes. Spleen cells or peripheral blood lymphocytes, previously incubated with antigen, are mixed with sheep red blood cells (SRBC). After addition of complement and incubation, plaques (clear areas) caused by the lysis of SRBC appear in the otherwise cloudy layer. Antibody forming cells can be detected by the appearance of plaques. The number of plaques obtained is proportional to the number of antibody producing lymphocytes in the cell population.

PROCEDURE

NMRI mice weighing 16–18 g or Lewis rats weighing 180–200 g of either sex are used.

Materials

- absorbed guinea pig complement
- SRBC stored in Alsever's solution

Positive Control

Spleen cells incubated with antigen and medium

Negative Control

Spleen cells incubated with medium alone. The animals are decapitated and the spleens are removed from the peritoneal cavity. A single cell suspension of 15×10^6 cells/ml is prepared. For the induction of PFC, a 0.5 ml splenocyte suspension is added to 0.5 ml of a suspension of SRBC, previously washed in medium and diluted to 8×10^6 cells/ml. Thereafter, 1 ml of the solution of the test compound is added and the limbrowells are incubated at 37°C in a CO_2 incubator for 5 days. Per group 3 limbrowells are set up. On day 5, the 3 wells of each group are pooled, washed in medium and the number of cells is determined. For each cell pellet, 875 μl of washed SRBC and 125 μl absorbed guinea pig complement are added. The suspension is mixed thoroughly and filled in chambers constructed of microslides. The chambers are placed in the

incubator at 37°C for 90–120 min. The plaque forming colonies are counted immediately after incubation.

EVALUATION

The activity of test compounds can be determined using the following formula:

1. PFC/3 wells:

$$x = \frac{\text{plaques} \times 100}{\mu\text{l}}$$

2. % change in the number of plaques:

$$x = \frac{\text{plaques} \times 100}{\text{plaquespos. control}}$$

$$d\% = x - 100$$

3. % change in number of cells:

$$x = \frac{\text{number of cells} \times 100}{\text{number of cells pos. control}}$$

$$d\% = x - 100$$

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1.2.1.6

Inhibition of Dihydro-Orotate Dehydrogenase

PURPOSE AND RATIONALE

Dihydro-orotate dehydrogenase catalyzes the fourth committed step in the de novo biosynthesis of pyrimidines. As rapidly proliferating human T cells have an exceptional requirement for de novo pyrimidine biosynthesis, small molecule dihydro-orotate dehydrogenase inhibitors constitute an attractive therapeutic approach to autoimmune diseases, immunosuppression, and cancer. The main mode of action of the immuno-suppressive compound leflunomide and its active metabolites is considered to be the inhibition of the enzyme dihydro-orotate dihydrogenase (Bruneau et al. 1998; Graul and Castañer 1998; Knecht and Löffler 1998; Rückemann et al. 1998; Schorlemmer et al. 1998; Herrmann et al. 2000; Liu et al. 2000).

PROCEDURE

A fragment of human dihydro-orotate dehydrogenase is expressed by means of the baculovirus expression vector system and purified to a specific activity greater than 50 U/mg (Knecht et al. 1996, 1997). Enzyme assays are performed with purified recombinant dihydro-orotate dehydrogenase at 30°C. The oxidation of the substrate dihydro-orotate and the reduction of the co-substrate quinone is coupled to the reduction of the chromogen 2,6-dichlorophenolindophenol (DCIP). The reaction mixture contains 0.1 mM Q_D or 0.1 M Q₁₀, 1 mM L-dihydro-orotate, 0.06 mM DCIP, 0.1% Triton X-100 in 50 mM Tris-HCl buffer, 150 mM KCl, pH 8.0. The reaction is started by addition of the enzyme. The loss of absorbance of the blue DCIP is monitored at 600 nm; $\epsilon = 18.8001 \text{ mol}^{-1} \text{ cm}^{-1}$. The enzyme activity in control assays without Q_D or Q₁₀ which is approximately 1% of maximum enzyme activity is subtracted from the activity values measured. Stock solutions of the test compounds are prepared in dimethylsulfoxide with further dilutions in the buffer taken for the assays.

EVALUATION

To determine the inhibitory potency of the agents, the initial velocity of dihydro-orotate dehydrogenase reaction is measured at saturating substrate concentrations, 1 mM dihydro-orotate and 100 μM Q_D and varying concentrations of the drugs (1 nM through 100 μM). The equation is fitted to the initial velocities:

$$v = V / \{1 + [I]/IC_{50}\}$$

([I] is the inhibitor concentration) in order to find the concentration causing 50% inhibition of the enzyme activity (IC_{50}).

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I.2.1.7**Sphingosine 1-Phosphate****I.2.1.7.1****General Considerations**

Sphingolipids have emerged as molecules whose metabolism is regulated to generation of bioactive products including ceramide, sphingosine, and sphingosine-1-phosphate. The balance between cellular levels of these bioactive products is recognized to be critical to cell regulation and may be a promising approach to tumor therapy (Huwiler and Pfeilschifter 2006); whereby ceramide and sphingosine cause apoptosis and growth arrest phenotypes, and sphingosine-1-phosphate mediates proliferative and angiogenic responses. Sphingosine kinase is a key enzyme in modulating the levels of these lipids (Hannun and Obeid 1995; Hofmann and Dixit 1998; Mathias et al. 1998; Prieschl et al. 1999; Pyne and Pyne 2000; Cummings et al. 2002; MacKinnon et al. 2002; Rosen and Liao 2003; Chen et al. 2004; Deguchi et al. 2004; Lee et al. 2004; Peng et al. 2004; Cyster 2005; Kee et al. 2005; Watterson et al. 2005; Gardell et al. 2006; Taha et al. 2006). Ceramide formation and degradation are influenced by nitric oxide (NO) (Huwiler et al. 1999a, 1999b; Franzen et al. 2002a, 2002b).

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1.2.1.7.2

Binding to Sphingosine 1-Phosphate Receptors

PURPOSE AND RATIONALE

At least five subtypes of the sphingosine 1-phosphate receptor with tissue specificity are known (Meyer zu Heringdorf et al. 1998; Kon et al. 1999; Im et al. 2000, 2001; Forrest et al. 2004; Hale et al. 2004a; Sanna et al. 2004; Zhou and Murthy 2004; Xin et al. 2004; Lepley et al. 2005; Kimura et al. 2006; Kitano et al. 2006).

The immunomodulator FTY720 is an agonist to sphingosine 1-phosphate receptors (Brinkmann et al. 2002; Gräler and Goetzl 2004; Kunzendorf et al. 2004; Xin et al. 2004; Albert et al. 2005; Bandhuvula et al. 2005; Chiba 2005; Sawicka et al. 2003, 2005; Habicht et al. 2005; Xin et al. 2006; Zhou et al. 2006). FTY720 is derived from ISP-1 (myriomycin), a fungal metabolite that is an eternal youth nostrum in traditional Chinese herbal medicine (Fujita et al. 1994). The compound {2-amino-2-[2-(4-octophenyl)ethyl]propane-1,3-diol} is a highly potent immune modulating agent.

Further derivatives such as sphingosine 1-phosphate receptor agonists (Hale et al. 2004b, 2004c; Clemens et al. 2005; Foss et al. 2005; Kiuchi et al. 2005; Jo et al. 2005; Li et al. 2005; Colandrea et al. 2006) and antagonists (Davis et al. 2005) have been described. Brinkmann et al. (2002) used the [γ -³⁵S]GTPS binding assay to study the binding of the immune modulator FTY720 to sphingosine 1-phosphate receptors.

Forrest et al. (2004) studied the binding of sphingosine 1-phosphate agonists on distinct receptor subtypes.

PROCEDURE

Receptors and Cell Lines

CHO cells stably expressing human S1P_{1,2,3,4,5} were used (Mandala et al. 2002). cDNA sequences encoding rodent S1P receptors were cloned from genomic DNA by polymerase chain reaction using the following primers for each respective receptor:

5'-GAACCCGGGTGTCCACTAGCATCCCGG and 5'-CCCGAATTCTTAGGAAGAAGAATTGACGTTTCC (mouse S1P₁), 5'-GAACCCGGGCGGCTTATAC TCAGAGTACC and 5'-GGCGAATTCTCAGACCAC TGTGTTACCCTC (mouse S1P₂), 5'-GAACCCGGG CAACCACGCATGCGCAGG and 5'-GTGCAATTC TCACTTGCAGAGGACCCCG (mouse S1P₃), 5'-GA

ACCCGGGAACATCAGTACCTGGTCCACGC and GCGGAATTCTAGGTGCTGCGGACGCTGG (mouse S1P₄), 5'-GAACCCGGGCTGCTGCGGCC GG and 5'-CGGAATTCAGTCTGTAGCAGTAGG CACC (mouse S1P₅), 5'-GTAGGATCCGTGTCCTC CACCAGCATC and 5'GGCCGAATTCTTAAGAAG AAGAATTGACGTTTC (rat S1P₁), 5'-GAACCCGG GCATCCACGCATGCGCAG and 5'-GCCGAATTC TCACCTGCAGAGGACCCCATCTG (rat S1P₃).

The polymerase chain reaction products were inserted in-frame after a FLAG tag using vector pCMV-Tag2 (Stratagene, La Jolla, Calif., USA). Stable lines were established by transfecting plasmids into CHO cells using lipofectamine reagent, selecting for neomycin resistance, and screening single cell cultures for increased [³³P]S1P-specific binding. Membranes were prepared from positive clones and confirmed in [³³P]S1P and [³⁵S]GTPγS binding assays.

S1P Receptor Assays

Binding assays were conducted as described by Mandala et al. (2002). [³³P]S1P was sonicated with fatty-acid-free bovine serum albumin, added to test compounds diluted in dimethylsulfoxide (DMSO), and mixed with membranes in 200 μl in 96-well plates with assay concentrations of 0.1 nM [³³P]S1P (22,000 dpm), 0.5% bovine serum albumin, 50 mM HEPES-Na (pH 7.5), 5 mM MgCl₂, 1 mM CaCl₂, and 0.3 to 0.7 μg of membrane protein. Binding was performed for 60 min at room temperature and terminated by collecting the membranes onto GF/B filter plates with a Packard Filtermate Universal Harvester. Filter bound radionuclide was measured on a Perkin Elmer 1450 MicroBeta. Specific binding was calculated by subtracting radioactivity that remained in the presence of 1000-fold excess of unlabeled S1P.

To measure functional activation of the S1P receptors, [³⁵S]GTPγS binding was measured. Membranes (1–4 μg of protein) were incubated in 96-well plates with test compounds diluted in DMSO in 100 μl of buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, and 2–10 μM GDP, depending on the expressed receptor. The assay was initiated with the addition of 100 μl of [³⁵S]GTPγS (1200 Ci/mmol or 44,400 Bq/mmol; Perkin Elmer Life and Analytical Sciences, Boston, Mass., USA) for an assay concentration of 125 pM. After 60 min of incubation at room temperature, membranes were harvested onto GF/B filter plates and bound radionuclides were measured.

MODIFICATIONS OF THE METHOD

Murata et al. (2000) described a radioreceptor-binding assay for quantitative measurement of sphingosine 1-phosphate.

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1.2.1.7.3

Sphingosine Kinase Activation Assay

PURPOSE AND RATIONALE

Sphingosine 1-phosphate produced by two sphingosine kinase isoenzymes, denoted SphK1 and SphK2, is the ligand for a family of specific G-protein-coupled receptors that regulate cytoskeletal re-arrangements and cell motility. Unlike the proliferative action of SphK1, the isoenzyme SphK2 has been shown to possess anti-proliferative and pro-apoptotic action. Both kinases have been cloned and functionally characterized (Kohama et al. 1998; Liu et al. 2000, 2003; Nava et al. 2000; Olivera 2000; Igarashi et al. 2003; Paugh et

al. 2003; Sanchez et al. 2003; Billich et al. 2005; Döll et al. 2005; Hait et al. 2005; Kharel et al. 2005; Okada et al. 2005; De Palma et al. 2006; Zemmann et al. 2006).

Sphingosine kinase activity assays were performed in a similar way by Paugh et al. (2003) and by Huwiler et al. (2006).

PROCEDURE

Sphingosine Kinase Activity Assay

In vitro kinase reactions were performed according to Olivera et al. (2000). In brief, 30 µg of protein lysates was incubated with 50 µmol/l of sphingosine (dissolved as 1 mmol/l stock solution in 4 mg/ml of BSA in PBS) and 10 µCi (370 kBq) of [γ -³²P]ATP for 15 min at 37°C. For SK-2 activity assay, the same buffer including 1 M KCl was used to inhibit SK-1 activity (Liu et al. 2000). Reactions were terminated by addition of 20 µl of 1N HCl followed by 800 µl of chloroform/methanol/HCl (100:200:1, v/v), 240 µl of chloroform and 240 µl of 2 mol/l KCl. After vigorous vortexing and phase separation, 50 µl of the lower organic phase was loaded onto TLC plates and run in 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v).

EVALUATION

Spots corresponding to S1P were analyzed and quantified using an Imaging System (Fuji).

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I.2.1.7.4

Lymphocyte Trafficking After Sphingosine 1-Phosphate Receptor Agonists

PURPOSE AND RATIONALE

Adaptive immunity depends on T-cell exit from the thymus and T and B cells traveling between secondary lymphoid organs to survey for antigen. After activation in lymphoid organs, T cells must again return to circulation to reach sites of infection. The immunomodulatory drug FTY720 induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing via the S1P receptor 1 (Chiba et al. 1998; Yanagawa et al. 1998a, 1998b; Henning et al. 2001; Forrest et al. 2004; Matloubian et al. 2004; Hait et al. 2005; Kharel et al. 2005; Huwiler et al. 2006). Mandala et al. (2000) described alteration of lymphocyte

trafficking by sphingosine 1-phosphate receptor agonists.

PROCEDURE

Induction of Lymphopenia and Reduction of Thoracic Duct (TD) Lymphocytes by S1P and Analogs in Rats

Blood or thoracic duct lymphocyte counts were determined by autoanalyzer (H2000, CARESIDE, Culver City, Calif., USA) and normalized to counts in vehicle controls after administration of FTY720 (2.5 mg/kg p.o.), or test compound. S1P was administered by continuous infusion beginning at 8 mg/kg per hour for 20 min followed by 2 mg/kg per hour for a further 220 min. The measured physiological S1P concentration in rat plasma by LC-MS was 0.5 µg/ml. This rose to a C_{max} of 2.5 µg/ml at 30 min and was maintained at 1.5 µg/ml for the remainder of the experiment. Studies on the effect on lymphocyte numbers in thoracic-duct-cannulated rats were performed after administration of FTY720 or test compound. Lymph flow remained constant for the duration of the experiment and numbers are shown as the average cell concentration maintained over the preceding 30 min.

FACS Measurement of Peripheral Blood Lymphocyte Depletion in Cannulated Rats:

Percentage depletion by FTY720 compared to vehicle control was measured. Similar nadir lymphopenia was produced by FTY720 or non-metabolizable phosphonates. Peripheral blood samples were diluted 1:1 with phosphate-buffered saline (PBS), layered on the same volume of Lymphocyte Separation Medium (ICN Biomedicals, Aurora, Ohio, USA) and centrifuged at 400 g for 30 min. Peripheral blood mononuclear cells (PBMC) were resuspended in PBS and counted using a hemocytometer. PBMC were then stained with FITC-labeled anti-CD8, PE-labeled anti-CD45RA, and Cy-Chrome-labeled anti-CD4 antibodies. Numbers of CD4-, CD8- and CD45RA-positive cells were calculated by multiplying total PBMC count with the percentages of CD4⁺, CD8⁺ and CD45RA⁺ generated from flow cytometry.

Quantitation of Lymph Node Cells

Single-cell suspensions were prepared by passage of tissues through a 40-µm sieve. Peripheral blood lymphocytes were further isolated from spleens by ammonium chloride lysis of red blood cells. Cells were subsequently washed in UltraCULTURE medium (Biowhittaker, Walkersville, Md., USA) and all samples were adjusted to the same volume with PBS. An equal volume of 4% paraformaldehyde was added

while gently vortexing the samples. The total number of viable, unstained lymphocytes per sample was determined by flow cytometry (FACScan; Becton Dickinson) using CELLquest software (Becton Dickinson), based upon forward and side scatter characteristics. Beads (Sigma; P7458) were used as an internal standard.

EVALUATION

Data were calculated as cell number per node by dividing the total number of lymphocytes quantitated by the number of nodes harvested per site (i. e., the number of Peyer's patches, mesenteric or peripheral lymph nodes collected).

MODIFICATIONS OF THE METHOD

Kawa et al. (1997) reported inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate.

Fueller et al. (2003) described activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate.

Roviezzo et al. (2004) studied human eosinophil chemotaxis and selective *in vivo* recruitment by sphingosine 1-phosphate.

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- New Zealand black/white F1 (B/W) mouse** (Helyer and Howie 1963; Kessler 1968): These animals develop nephritis similar to that in human systemic lupus erythematosus and show mononuclear cell infiltration in salivary and lachrymal glands such as in human Sjögren's syndrome.
- A substrain of the autoimmune-prone mouse, NZB/kl**, was found to show spontaneous elevation of the auditory brainstem response threshold with age (Sone et al. 1995).
- Immunodeficient alymphoplasia mice** were recommended as a spontaneous model for Sjögren's syndrome (Tsubata et al. 1996). Mice homozygous for an autosomal recessive mutation aly (alymphoplasia) lack both lymph nodes and Peyer's patches and show defects in both humoral and cellular immunity. Histopathological analyses revealed chronic inflammatory changes in exocrine organs such as the salivary gland, lacrimal gland and the pancreas.
- The Palmerston North autoimmune mouse strain** which exhibits both spontaneous systemic autoimmune disease and otic capsule bone formation has been proposed as a model for otic capsule osteogenesis and otosclerosis (Hertler and Trune 1990; Traynor et al. 1992).
- In aging **BDF1 mice**, Hayashi et al. (1988) described spontaneous development of autoimmune sialadenitis.
- Robison et al. (1994) examined the relationship between orchitis and aspermatogenesis in various strains of H₂ congenic mice and defined a genetic predisposition to spontaneous aspermatogenesis.
- Motheaten mice.** Mice homozygous for the autosomal recessive motheaten (me) or the allelic viable motheaten (me^v) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Su et al. 1998).

I.2.2

In Vivo Methods for Testing Immunological Factors

I.2.2.1

Spontaneous Autoimmune Diseases in Animals

Several spontaneous autoimmune diseases have been reported in several inbred animal strains:

New Zealand black mouse (NZB mouse) (Biel-schowski et al. 1959; Howie and Helyer 1968;

Barthold et al. 1974; Blanchard and Bach 1980). The NZB mouse develops a spontaneous autoimmune disease with autoimmune hemolytic anemia, splenomegaly, glomerulonephritis, lymphoproliferative disorders and peptic ulcerations.

New Zealand black/white F1 (B/W) mouse (Helyer and Howie 1963; Kessler 1968): These animals develop nephritis similar to that in human systemic lupus erythematosus and show mononuclear cell infiltration in salivary and lachrymal glands such as in human Sjögren's syndrome.

A substrain of the autoimmune-prone mouse, NZB/kl, was found to show spontaneous elevation of the auditory brainstem response threshold with age (Sone et al. 1995).

Immunodeficient alymphoplasia mice were recommended as a spontaneous model for Sjögren's syndrome (Tsubata et al. 1996). Mice homozygous for an autosomal recessive mutation aly (alymphoplasia) lack both lymph nodes and Peyer's patches and show defects in both humoral and cellular immunity. Histopathological analyses revealed chronic inflammatory changes in exocrine organs such as the salivary gland, lacrimal gland and the pancreas.

The Palmerston North autoimmune mouse strain which exhibits both spontaneous systemic autoimmune disease and otic capsule bone formation has been proposed as a model for otic capsule osteogenesis and otosclerosis (Hertler and Trune 1990; Traynor et al. 1992).

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Robison et al. (1994) examined the relationship between orchitis and aspermatogenesis in various strains of H₂ congenic mice and defined a genetic predisposition to spontaneous aspermatogenesis.

Motheaten mice. Mice homozygous for the autosomal recessive motheaten (me) or the allelic viable motheaten (me^v) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Su et al. 1998).

The genetic, hormonal and behavioural influence on spontaneously developing arthritis in normal mice has been reviewed by Holmdahl et al. (1992).

Non-obese diabetic mouse (NOD mouse) (Makino et al. 1980; Miyazaki et al. 1985; Leiter et al. 1987). The inbred NOD mouse is considered a good model for type I diabetes mellitus. Mononuclear cells infiltrate the pancreatic islets of Langerhans from 6–8 weeks of age, followed by a progressive and

selective destruction of insulin-producing β -cells and the onset of IDDM from the 12th week of age onwards.

Itoh et al. (1997) studied the requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice.

Quartey-Papafio et al. (1995) showed that aspartate at position 57 of nonobese diabetic I-A(g7) β -chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus in the NOD mouse.

The NOD mouse was also recommended to study the pathogenesis of autoimmune thyroiditis (Many et al. 1996).

Bio-breeding rat (BB rat) (Like et al. 1982; Field 1983; Yale and Marliss 1984). On the basis of clinical and histopathological parameters, the BB rat is considered a useful model for human IDDM. The disease in the BB Rat is characterized by infiltration of lymphocytes and macrophages into the islets of Langerhans.

Allen and Thupari (1995) described spontaneous autoimmune lymphocytic thyroiditis in *BB/Wor rats*.

Obese strain chicken (OS chicken) (van Tienhoven and Cole 1962; Cole 1966; Cole et al. 1968, 1970; Wick et al. 1974). The OS chicken is perhaps the best studied model for an organ-specific, spontaneously occurring autoimmune disease, viz. spontaneous autoimmune thyroiditis, which closely resembles human Hashimoto thyroiditis. The spontaneous autoimmune thyroiditis in obese chicken was further studied by Neu et al. 1986; Kroemer et al. 1989; Cihak et al. 1995; Hala et al. 1996; Dietrich et al. 1997.

Chickens of the University of California line 200 (**UCD-200 chickens**) develop an inherited inflammatory fibrotic disease that closely resembles human progressive systemic sclerosis (scleroderma) (Gershwin et al. 1981; Van de Water et al. 1984; Brezinscheck et al. 1993).

Schumm-Draeger and Fortmeyer (1996) described **autoimmune thyroiditis in the cat** as a spontaneous disease model.

Spontaneous autoimmune thyroiditis was found in **Mastomys** (*Praeomys coucha*) by Solleveld et al. (1985) and recommended as an animal model of human disease.

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I.2.2.2

Acute Systemic Anaphylaxis in Rats

PURPOSE AND RATIONALE

Rats are immunized with ovalbumin and *Bordetella pertussis* suspension as adjuvant. After 11 days the animals are challenged by intravenous injection of ovalbumin. The shock symptoms can be inhibited by corticosteroids and intravenous disodium cromoglycate.

PROCEDURE

Female Sprague-Dawley rats weighing 120 g are immunized by i.m. injection of 10 mg/kg highly purified ovalbumin. Simultaneously 1 ml of *Bordetella pertussis* suspension (2×10^{10} organisms) is injected intraperitoneally. IgE antibodies are induced and attached to the surface of mast cells and basophilic granulocytes. Eleven days later the animals are challenged by intravenous injection of 25 mg/kg highly purified ovalbumin. This results in formation of antigen-antibody-complexes on the surface of mast cells and basophilic granulocytes in blood and in all organs with immediate release of various mediators of anaphylaxis, such as histamine, serotonin, SRS-A, prostaglandins; in shock symptoms and 80% lethality. Corticosteroids, e.g. dexamethasone 1–10 mg/kg s.c. are given 18 h prior to challenge, or 30 mg/kg disodium cromoglycate i.v. before injection of ovalbumin. Ten–20 animals are used for each group.

EVALUATION

The shock symptoms are scored and mortality counted. Results after treatment are compared with untreated controls. Pretreatment with corticosteroids or disodium cromoglycate can inhibit death and ameliorate shock symptoms. Statistical calculation is performed using the χ^2 -test.

MODIFICATIONS OF THE METHOD

Desensitization by repeated 'microshocks' of constant strength in guinea pigs has been reported by Herxheimer (1952).

Acute systemic anaphylaxis experiments have also been performed in guinea pigs and in mice. In guinea pigs anaphylactic bronchospasm can be measured with the Konzett and Rössler method (see D.2.2.1) (Davies and Evans 1973).

Moreover, anaphylactic bronchospasm can be measured in isolated guinea pig lungs according to the method of Bhattacharya and Delaunoy (1955).

Anaphylaxis can be measured in the chopped guinea pig lung by assay of the supernatant in the isolated guinea pig ileum in the presence of 2×10^{-7} M atropine (Austen and Brocklehurst (1961).

Ufkes and Ottenhof (1984) sensitized Brown-Norway rats with a suspension of trinitrophenyl haptenized ovalbumin together with AlPO_4 as adjuvant. Bronchial and cardiovascular function were studied after treatment with anti-allergic agents and antigen challenge.

Elwood et al. (1992) studied the effect of dexamethasone and cyclosporin A on allergen-induced airway hyperresponsiveness and inflammatory cell responses in sensitized Brown-Norway rats.

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mediators, e. g. histamine, which induce contraction in isolated ileum.

PROCEDURE

Guinea pigs of either sex weighing 300–350 g are sensitized with alum precipitated egg albumin. Alum egg albumin is prepared by dissolving egg albumin (1 mg/ml) in six percent aluminum hydroxide gel, suspended in saline. The mixture is stirred and kept at room temperature. Each animal receives at the same time injections of 0.125 ml of this mixture in each foot pad and 0.5 ml subcutaneously. After 4 weeks the animals are killed and the ileum is dissected out. Cleaned pieces, about 2–3 cm long, are mounted in an organ bath containing Tyrode solution at 37°C. The strips are allowed to equilibrate for 15 min. The contractility of the ileum strips is tested by adding 10^{-4} g/ml BaCl_2 solution. To one organ bath the standard (2×10^{-6} g/ml final concentration of Tribenosid = 1-O-ethyl-3,5,6-tri-O-benzyl-D-glucofuranoside = Glyvenol CIBA) and to other vials the test compounds (final concentration up to 10^{-5} g/ml) are added. One organ bath serves as control. After 3 min ovalbumin in a final concentration of 2×10^{-6} g/ml is added. The contractions are recorded with strain gauges by a polygraph.

EVALUATION

The results are expressed as presence or absence of blocking activity (percentage inhibition). If anti-anaphylactic activity is observed, ED_{50} values using different doses are calculated.

CRITICAL ASSESSMENT OF THE METHOD

Positive results can also be achieved with spasmolytics, local anesthetics, antihistaminics, and sympathicomimetics.

MODIFICATIONS OF THE METHOD

The method has been modified by testing histamine release in the lung after challenging with egg albumin. Either lung strips from sensitized guinea pigs are suspended in an organ bath and their contractions are measured after addition of egg albumin or the entire lung tissue is dissected out and washed free from blood by perfusing with warm oxygenated Tyrode solution via the pulmonary artery. The lung tissue is chopped and washed with Tyrode solution in order to remove the remaining blood. The chopped lung tissue is divided into 24 samples, each of approximately 100 mg wet weight. These are incubated at 37°C in Tyrode solution for 15 min with continuous agitation by rocking, after which, 1 mg/ml of egg albumin is added to

1.2.2.3**Anti-Anaphylactic Activity (Schultz–Dale Reaction)****PURPOSE AND RATIONALE**

Guinea pigs are sensitized against egg albumin. Challenge after 3 weeks causes in isolated organs release of

the reaction mixture. After shaking for 10 min at 37°C, the supernatant is collected and assayed for histamine with guinea pig ileum. Atropine sulfate 2 mg/ml is added in Tyrode solution. The residual histamine is obtained by boiling the tissue in 5 ml Tyrode solution for 10 min. The tubes are then placed on ice for 1 h to allow complete diffusion. Released histamine is expressed as a percentage of total histamine content.

Koppel et al. (1981) developed a method to induce contraction of immunologically sensitized mouse trachea by antigen (Schultz–Dale reaction).

The trachea of sensitized guinea pigs was used by Omote et al. (1994).

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I.2.2.4

Passive Cutaneous Anaphylaxis

PURPOSE AND RATIONALE

Passive cutaneous anaphylaxis is a immune reaction of the immediate type. By passive immunization of rats in the skin with rat anti-ovalbumin serum and a challenge 2 days later with ovalbumin at the same skin area antigen-antibody complexes are formed in the mast cells inducing release of mediators. This results in vasodilatation, increase in permeability of the vessel walls and leakage of plasma. To make the allergic reaction visible, Evan's blue dye is administered along with the antigen. Evan's blue dye is attached to the albumin fraction of plasma, producing a blue spot. This blue spot indicates that an anaphylactic reaction has taken place in the skin.

PROCEDURE

For preparation of antiserum male rats weighing 200–250 g are adrenalectomized and are allowed to recover for 3 days. Thereafter, animals are sensitized with egg albumin (1 mg/animal) using aluminium hydroxide gel (200 mg) as adjuvant. Alum egg albumin is prepared by dissolving 1 mg/ml of egg albumin in 20% aluminium hydroxide gel, suspended in saline. Each animal simultaneously receives 0.125 ml of the above solution in each foot pad and 0.5 ml subcutaneously. After 8 days, the animals are bled and antiserum is collected.

For the test, the antiserum is diluted in such a manner as to give a wheal of 15–20 mm diameter in a preliminary titration. Aliquots of 100 µl of appropriate dilution of antiserum are injected intradermally into the shaved dorsal skin of normal male rats weighing about 100 g. After 24 h of latent period each animal is challenged with the intravenous administration of 0.1 ml of 2.5% Evans blue dye containing 25 mg/ml of egg albumin. In the case of intravenous administration, the test compound is administered simultaneously with the antigen and the dye. In case of oral testing, the compound is given orally 1 h prior to challenge. The animals are sacrificed 30 min after the challenge. The amount of Evans blue dye leaked at the site of passive cutaneous anaphylactic reaction is extracted and determined colorimetrically at 620 µm wavelength.

EVALUATION

The amount of Evans blue extracted from passive cutaneous anaphylactic reaction is taken as 100 percent. Percent inhibition of passive cutaneous anaphylactic reaction in the rats treated with the test compound is calculated. The standard disodium cromoglycate at a dose of 3 mg/kg i.v. or 30 mg/kg orally results in 80–100% inhibition. Using different doses, ED_{50} values can be calculated.

MODIFICATIONS OF THE METHOD

Goose and Blair (1969) used *Bordetella pertussis* and extracts of the worm *Nippostrongylus brasiliensis* as antigens in passive cutaneous anaphylaxis experiments in the rat.

Patterson et al. (1971) tested passive cutaneous reactivity to antihuman IgE in rhesus monkeys.

Without immunization, plasma extravasation after bradykinin injection can be tested in anesthetized Sprague-Dawley rats (Lembeck et al. 1991). Evans blue dye is injected to stain plasma proteins. After injection of bradykinin antagonists followed by bradykinin injection, the rats are perfused with phys-

iological saline. The trachea, the urinary bladder, and the duodenum are resected, weighed and incubated for 48 h in formamide at 50°C (Saria et al. 1983). The amount of Evans blue extracted is measured photometrically at 620 nm.

Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea pigs using pontamine sky blue 6X as indicator were studied by Miles and Miles (1952).

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1.2.2.5

Arthus Type Immediate Hypersensitivity

PURPOSE AND RATIONALE

The immune complex induced Arthus reaction comprises inflammatory factors that have been implicated in the acute responses in joints of rheumatic patients. Complement and polymorphonuclear neutrophils are activated via precipitating antigen-antibody complexes leading to an inflammatory focus characterized by edema, hemorrhage and vasculitis. Arthus reaction of the immediate type becomes maximal 2–8 h after challenge.

PROCEDURE

Ovalbumin Suspension

1700 mg ovalbumin are suspended in 100 ml paraffin oil. 4.38 ml pertussis vaccine are suspended in 70 ml

0.9% NaCl-solution. Both suspensions are mixed to form an emulsion.

Wistar or Sprague-Dawley rats of either sex weighing 220–280 g can be used. Seven days prior to start of the experiment rats are sensitized by i.m. administration of 0.5 ml of the ovalbumin suspension. They are housed in groups of eight with standard food and water ad libitum.

Twenty-four hours and one hour prior to induction of the Arthus reaction, test compounds are administered to groups of 8 animals. The rats are challenged by injection of 0.1 ml of 0.04% solution of highly purified ovalbumin in the left hind paw. Swelling of the paw occurs which reaches a maximum after a few hours. The footpad thickness can be measured by calipers. One group of sensitized animals treated with solvent alone serves as positive control, one group of non-sensitized animals treated with solvent alone serves as negative control. Standard doses are 30 mg/kg cortisone or 10 mg/kg prednisolone p.o.

EVALUATION

The change in footpad thickness is expressed as the percent change from the vehicle control group. Comparison of experimental group to positive control is evaluated statistically using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Instead of ovalbumin, sheep red blood cell suspensions can be used for immunization and for challenge in mice (Omote et al. 1994).

Nagakawa et al. (1990) sensitized mice by s.c. injection of bovine serum albumin in complete Freund's adjuvant and boosted on day 21 by an intradermal injection of BSA. On day 28, the Arthus reaction was elicited by intradermal injection of BSA. Four hours later, an erythematous skin reaction over an area of more than 8 mm² was regarded as positive.

Kamei et al. (1991) immunized guinea pigs by injection of a mixture of egg albumin and Freund's complete adjuvant subcutaneously into the food pad or i.m. into the hind leg. The injection was repeated 4 times at 7 day intervals. Ten days after the last immunization, 0.2 ml of 2.5% egg albumin was injected sc. into the dorsal skin of the animals. The intensity of the Arthus reaction was evaluated by measuring the inflamed area according to scores.

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I.2.2.6

Delayed Type Hypersensitivity

PURPOSE AND RATIONALE

Delayed type hypersensitivity is a reaction of cell mediated immunity and becomes visible only after 16–24 h. The same methods as for testing immediate type hypersensitivity can be used.

PROCEDURE

Rats are sensitized in the same way by i.m. administration of 0.5 ml ovalbumin suspension 7 days prior to the start of the experiment as described for testing immediate type hypersensitivity. They are challenged by injection of 0.1 ml of 0.04% solution of highly purified ovalbumin in the left hind paw. Footpad thickness is measured immediately and 24 h after ovalbumin administration.

MODIFICATIONS OF THE METHOD

Mizukoshi et al. (1994) injected female CDF1 mice intradermally with a suspension of 2×10^8 sheep red blood cells/50 μ l into the left foot pad. A second booster of the same dose was given to the right foot pad on day 4. The thickness of the foot pads was measured on the following day, and the difference in the thickness between the right and the left food pads was taken as the degree of swelling.

Kamei et al. (1991) immunized mice by applying 0.15 ml of 7% picryl chloride/ethanol solution to the skin of the shaved abdomen. The second immunization was performed 6 days later. One week after the second immunization, 1 drop of 1% picryl chloride olive oil solution was applied to the ear and the thickness of the ear was measured by a thickness gauge 24 h later.

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I.2.2.7

Reversed Passive Arthus Reaction

PURPOSE AND RATIONALE

In the reversed passive Arthus reaction the antigen is injected intravenously followed by a local injection – either intradermally or into the pleural space – of the respective antibody. Generation of an immune-mediated reverse passive Arthus reaction in the rat pleural cavity results in a classic acute inflammatory response. The methods are used to evaluate new anti-inflammatory agents.

PROCEDURE

Male Lewis rats weighing 200–250 g are fasted overnight prior to use with free access to water. The animals receive 5 mg bovine serum albumin in 0.2 ml sterile saline intravenously, followed 30 min later by injection of 1 mg rabbit anti-BSA in 0.2 ml sterile saline into the right pleural cavity under light halothane anesthesia. Drugs or vehicle controls are administered by gastric gavage in 1 ml/100 g body weight at different times prior to the anti-BSA. The animals are sacrificed at various intervals after anti-BSA injections by CO₂ inhalation (after 5 min for thromboxane B₂ determination, after 10 min for leukotriene B₄ determination, and after 4 h at the peak time of neutrophil infiltration). The fluid exudate is removed from the pleural cavity by gentle vacuum aspiration and the volume is recorded. Eicosanoids in the pleural exudate are quantitated by commercial RIA kits.

EVALUATION

The values after treatment with various doses of test compounds are compared with those of vehicle controls.

MODIFICATIONS OF THE METHOD

The antibody can be injected intradermally into the shaved skin of rats after intravenous injection of the antigen (e. g., human albumin) together with Evans blue dye solution. Extravasated dye is determined in skin punches (Camussi et al. 1990; Burch et al. 1992; Okamoto et al. 1992).

Bailey and Sturm (1983) induced the reverse passive Arthus reaction in rats using bovine serum albumin as antigen into the tail vein and rabbit anti-bovine serum albumin into the skin site. One hour after oral dosing with vehicle or drug, animals were lightly anesthetized and their hair was shaved from the mid-dorsal region with electric clippers. Each animal was injected intradermally with 40 μ l on the left side of the mid-dorsal line and with 40 μ l of rabbit anti-bovine serum albumin (5.0 mg/ml antibody protein), diluted 1:4 with phosphate-buffered saline on the right side of the dorsal midline. Immediately following the intradermal challenge, each rat received 0.5 ml phosphate-buffered saline containing 1.0 mg bovine serum albumin injected in the tail vein. Four hours after intradermal challenge, the animals were sacrificed. The full thickness skin was removed from the back and discs 8 mm in diameter were punched out with a metal punch. Wet weight of the samples from the phosphate-buffered saline- and antibody-injected site was determined and the edema induced by the reverse passive Arthus reaction calculated as the difference between both weights.

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1.2.2.8**Adjuvant Arthritis in Rats****PURPOSE AND RATIONALE**

Adjuvant arthritis in rats has been described by Pearson and Wood (1959) exhibiting many similarities to human rheumatoid arthritis. Injections of complete Freund's adjuvant into the rat paw induces inflammation as primary lesion with a maximum after 3 to 5 days. Secondary lesions occur after a delay of approximately 11 to 12 days which are characterized by inflammation of non-injected sites (hindleg, forepaws, ears, nose and tail), a decrease of weight and immune responses. The procedure has been modified by several authors in order to differentiate between anti-inflammatory and immunosuppressive activity (e. g. Perper et al. 1971). Anti-inflammatory compounds do not inhibit secondary lesions, which are prevented or diminished by immunosuppressive agents. Two protocols, termed "preventative" (or "prophylactic") and "therapeutic" (or "established") adjuvant arthritis, have gained wide usage for assessing a drug's potential anti-arthritic activity (Schorlemmer et al. 1999).

PROCEDURE

The choice of the animal strain has been found to be very important for the performance of this test.

Wistar–Lewis rats have been proven to be very suitable in contrast to other sub strains. Male rats with an initial body weight of 130 to 200 g are used. On day 1, they are injected into the sub plantar region of the left hind paw with 0.1 ml of complete Freund's adjuvant. This consists of 6 mg mycobacterium butyricum (Difco) being suspended in heavy paraffin oil (Merck) by thoroughly grinding with mortar and pestle to give a concentration of 6 mg/ml. Dosing with the test compounds or the standard is started on the same day and continued for 12 days. Paw volumes of both sides and body weight are recorded on the day of injection, whereby paw volume is measured plethysmographically with equipment as described in the paw edema tests. On day 5, the volume of the injected paw is measured again, indicating the primary lesion and the influence of therapeutic agents on this phase. The severity of the induced adjuvant disease is followed by measurement of the non-injected paw (secondary lesions) with a plethysmometer. Purposely, from day 13 to 21, the animals are not dosed with the test compound or the standard. On day 21, the body weight is determined again and the severity of the secondary lesions is evaluated visually and graded according the following scheme:

| | | score |
|------------|-----------------------------------------|-------|
| ears: | absence of nodules and redness | 0 |
| | presence of nodules and redness | 1 |
| nose: | no swelling of connective tissue | 0 |
| | intensive swelling of connective tissue | 1 |
| tail: | absence of nodules | 0 |
| | presence of nodules | 1 |
| forepaws: | absence of inflammation | 0 |
| | inflammation of at least 1 joint | 1 |
| hind paws: | absence of inflammation | 0 |
| | slight inflammation | 1 |
| | moderate inflammation | 2 |
| | marked inflammation | 3 |

EVALUATION

- For primary lesions: The percent inhibition of paw volume of the injected left paw over vehicle control is measured at day 5.
- For secondary lesions: The percentage inhibition of paw volume of the non-injected right paw over controls is measured at day 21.
- An arthritic index is calculated as the sum of the scores as indicated above for each animal. The average of the treated animals is compared with the control group.
- The total percentage change is calculated as follows by addition of:

percent inhibition of the injected paw on day 5 + percent inhibition of the non-injected paw on day 21 + percent change of the arthritic index.

Doses of 0.3 mg/kg indomethacin p.o. and 20–50 mg/kg phenylbutazone p.o. are effective on the primary lesions when dosage is started at the day of injection of the irritant. They are not effective on the secondary lesions.

In contrast, immunosuppressants like cyclophosphamide at a dose of 7 mg/kg inhibited the secondary lesions even when started at day 9 or later.

CRITICAL ASSESSMENT OF THE METHOD

Evidence was given that adjuvant arthritis in the rat is associated with chronic pain (Colpaert 1987) The measure of pain in this model still presents some technical problems since the evaluation is based on the somewhat biased observation of the behavioral responses.

MODIFICATIONS OF THE METHOD

A review was given by Gardner (1960) on the experimental production of arthritis.

Moran et al. (1999) compared adjuvant arthritis and selected animal models of arthritis to rheumatoid arthritis with special emphasis on the mechanism of joint destruction.

Kazuna and Kawai (1975) and Rooks et al. (1982) used rats with established lesions to test analgesics in the arthritic flexion pain test. The method is claimed to be specific by detecting only central analgesics and nonsteroidal anti-inflammatory drugs but not other classes such as CNS-depressant or antihistaminic drugs.

Brackertz et al. (1977) established antigen-induced arthritis in the mouse by immunization with methylated bovine serum albumin in complete Freund's adjuvant with B pertussis vaccine.

A streptococcal cell wall-induced arthritis in rats has been described by Wilder et al. (1982, 1987) and Yocum et al. (1986).

Lewis et al. (1997) studied degradation of articular cartilage in a rat monoarthritis model induced by an intra-articular injection of *Propionibacterium acnes*.

Crossley et al. (1989) reported on a monoarticular antigen-induced arthritis in rabbits and mice.

α -2-Glycoprotein levels have been recommended as parameter for severity and inhibition of experimental immunoarthritis in the rat by Sandow et al. (1971).

Pircio et al. (1975) recommended a method for the evaluation of analgesic activity using adjuvant-induced arthritis in rats. The degree of vocalization

was recorded from 5 rats placed together in a counting chamber.

Cruwys et al. (1994) sensitized rats on day 0 and 7 with multiple intradermal injections of methylated bovine serum albumin emulsified in Freund's complete adjuvant. On day 21, the animals were challenged by the intra-articular injection of 100 μ l 0.5% solution of methylated bovine serum albumin into the right knee. The progress of the monoarticular arthritis was monitored by daily measurement of joint diameter.

Butler et al. (1991) described a limited arthritic pain model for chronic pain and inflammation studies using injections of 0.05 ml of complete Freund adjuvant into the left tibio-tarsal joint of Sprague-Dawley rats.

Issekutz et al. (1994) studied the role of tumor necrosis factor- α and IL-1 in polymorphonuclear leukocyte and T lymphocyte recruitment to joint inflammation in adjuvant arthritis.

Esser et al. (1995) measured radiographic changes in adjuvant-induced arthritis in rats by quantitative image analysis. Digitized radiographs of the calcaneus were examined for changes in the mean and in the distribution of gray values. Periosteal new bone formation was measured as an increase in image area of the calcaneus.

Mercuric chloride (HgCl_2) induces a syndrome of autoimmunity in Brown-Norway rats characterized by a variety of IgG antibodies, very high concentrations of serum IgE, proteinuria, leukocytoclastic vasculitis which predominantly affects the caecum, and an inflammatory polyarthropathy (Kiely et al. 1995, 1996).

Kawahito et al. (2000) reported that 15-deoxy- $\Delta^{12,14}$ -PGJ₂ which activates PPAR- α , induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. Cuzzocrea et al. (2002) found that prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ attenuates the development of acute and chronic inflammation.

Bolon et al. (2004) described a method for rapid quantification of intralesional osteoclasts in the hind paws of Lewis rats with adjuvant-induced arthritis. A 4- μ m-thick section of the decalcified hind paw was stained to demonstrate osteoclasts using an indirect immunoperoxidase method and a rabbit anti-human monoclonal antibody directed against the osteoclast marker cathepsin K, which is an osteoclast protease primarily responsible for the resorption of bone. The sections were evaluated using tiered, semi-quantitative criteria to grade bone erosions and intralesional osteoclasts.

Kong et al. (1999), Campagnuolo et al. (2002), and Bolon et al. (2002a, 2002b) used Lewis rats with ad-

juvant arthritis to describe the effects of osteoprotegerin, an endogenous antiosteoclast factor for protecting bone in rheumatoid arthritis.

Francischi et al. (2000) described anti-inflammatory and analgesic effects of the phosphodiesterase 4 inhibitor rolipram in the rat model of adjuvant-induced arthritis.

Boyle et al. (2001) reported anti-inflammatory effects of a non-nucleoside adenosine kinase inhibitor in rat adjuvant arthritis.

Fujisawa et al. (2002) demonstrated the effects of highly water-soluble matrix metalloproteinase inhibitors in a rat adjuvant-induced arthritis model.

Wei et al. (2004) described effects and mechanisms of a dual inhibitor of interleukin-1 and tumor necrosis factor on adjuvant arthritis in rats.

Boe et al. (1999) reported that interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis.

Gauldie et al. (2004) described a robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. DBA/1 and C57BL/6 male mice were injected intra-articularly into a stifle joint with FCA (5 μ g in 5 μ l) once per week for 4 weeks. Measurement of joint diameter and joint histopathology were used to monitor the course of arthritis. Inflammatory hyperalgesia was assessed as the pressure causing a limb withdrawal. Standard drugs, such as indomethacin or prednisolone, caused a decrease in joint inflammation and associated hyperalgesia.

Consden et al. (1971), Cook and Jasin (1972), Cook et al. (1972), Jasin and Cook (1977) produced a chronic experimental monoarthritis by intra-articular injection of antigens into previously immunized **rabbits**.

Henderson et al. (1990) induced monoarticular arthritis in ovalbumin-sensitized rabbits by intra-articular injection of ovalbumin (antigen-induced arthritis) or in naive rabbits by injecting hyaluronic acid mixed with the polycation poly-D-lysine (polycation-induced arthritis).

Arner et al. (1995) compared the alterations in proteoglycan metabolism in antigen-induced arthritis and polycation-induced arthritis in rabbits and determined the involvement of interleukin-1 in the cartilage degradation that occurs in these models of rheumatoid arthritis.

Lewthwaite et al. (1995) studied the antifibrotic action of interleukin-1 receptor antagonist in antigen-induced monoarticular arthritis in New Zealand White rabbits.

Arthritis occurs in **pigs** due to infection with *Erysipelothrix rhusiopathiae* (Ajmal 1969). Experimental erysipelothrix infection in pigs can be used as a model for rheumatism research (Schulz et al. 1975a, b, 1977). Infections are established by oral or parenteral administration of standardized serotype B erysipelas strains.

Erysipelothrix arthritis could also produced in rats and **rabbits** (White et al. 1975; Glynn 1977).

Arthritis due to infection with *Mycoplasma synoviae* occurs naturally among domestic poultry (Olson et al. 1954, 1964). Arthritis in **chickens** after mycoplasma infection has been used as experimental model (Kerr and Olson 1970; Cullen 1977).

Experimental models of arthritis due to streptococcal infections have been proposed for various species: **mice** (Cayeux et al. 1966; Hook et al. 1960; Ohanian et al. 1969), **rats** (Jasmin 1967; Koga et al. 1973), **rabbits** (Cecil et al. 1939; Cook and Fincham 1966; Ginsburg et al. 1968, 1977; Norlin 1960; Shimizu et al. 1958; Stein et al. 1973), **pigs** (Roberts et al. 1968, 1969).

Avridine-Induced Arthritis

The injection of avridine [*N,N*-dioctadecyl-*N',N'*-bis(2-hydroxyethyl) propanediamine / CP-20961], emulsified in Freund's adjuvant, at the base of the tail is arthritogenic in susceptible rat strains (Meacock et al. 1994; Brun et al. 1995; Vingsbo et al. 1995; Lorentzen and Klareskog 1997; Joe and Wilder 1999; Van Bilsen et al. 2004).

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I.2.2.9

Collagen Type II Induced Arthritis in Rats

PURPOSE AND RATIONALE

As reported by Trentham et al. (1977) intradermal injection of homologous or heterologous type II collagen in incomplete Freund's adjuvant results in an inflammatory polyarthritiden in rats. The demonstration of antibodies to collagen in patients with rheumatic polyarthritiden suggests that autoimmunity may contribute to the pathophysiology of synovitis and joint destruction. Because of the similarities of the symptoms in rats to human disease the test is considered to be useful to detect anti-inflammatory and immunosuppressive properties of test compounds.

PROCEDURE

Bovine type II collagen is prepared from nasal septum cartilage, which is cut into small fragments, frozen in liquid nitrogen, and pulverized in a freezer mill. Proteoglycans are extracted overnight by stirring 25 g of pulverized cartilage in 1 liter of 0.2 N NaOH. Following centrifugation at 20,000 g for 30 min, the residue is washed with 250 ml of absolute ethanol, the supernatant aspirated, and the residue vacuum dried. Hundred mg pepsin are added to 150 ml of 0.5 M acetic acid, after which 1.0 g of cartilage is added to reach a cartilage to pepsin ratio of 10:1 (w/w). The mixture is stirred 18 h at room temperature and centrifuged at 20,000 g for 1 h. Acid soluble collagen present in the supernatant is precipitated by adding NaCl to reach a final concentration of 0.9 M, followed by centrifugation at 20,000 g for 1 h. The precipitate from 1.0 g car-

tilage is dissolved in 100 ml 1.0 N NaCl/0.005 M Tris-HCl, pH 7.5, and stirred for 3 days. Then, the solution is dialyzed against 0.02 M Na₂HPO₄, pH 9.4, and the precipitate collected by centrifugation at 30,000 g for 1 h. The pellet is dissolved in 0.5 M acetic acid, dialyzed against 6 liters of 0.01 M acetic acid, and lyophilized. All procedures, unless otherwise stated are performed at 4°C.

Test procedure. Collagen is dissolved in a concentration of 2.0 mg/ml in 0.1 M acetic acid overnight at 4°C. This solution is added dropwise to an equal volume of chilled incomplete Freund's adjuvant. Six to 12 male Wistar rats with an initial weight of about 120 g are used for each group. On day 1, each rat receives a total of 0.5 mg collagen in 0.5 ml, equally divided, in 5 sites. All injections are intradermal, one at the base of each appendage and one in the nape of the neck. Seven days post-immunization, the animals receive identical booster injections. Control animals receive only the incomplete Freund's adjuvant diluted with 0.1 M acetic acid.

The volume of both hind paws is measured plethysmographically on day 20. To minimize the possibility of including animals with minimal transient disease, only animals with a paw volume of 1.8 ml or greater are used for further testing. From days 20–40, the animals receive the test compounds p.o. once a day. On day 41, the paw volumes are recorded again.

EVALUATION

The paw volumes of treated animals are recorded plethysmographically. The increase versus day 20 is calculated. The increase is compared with that of controls or animals treated with a standard drug. Otherwise, arthritic scores can be determined. Nonsteroidal anti-inflammatory drugs such as indomethacin in a dose of 2 mg/kg p.o. or phenylbutazone in a dose of 150 mg/kg p.o., but not acetylsalicylic acid in a dose of 50 mg/kg p.o. have been found to be active. Likewise, corticosteroids and immunosuppressives, but not D-penicillamine, were active.

CRITICAL ASSESSMENT OF THE METHOD

Non-steroidal and steroidal anti-inflammatory compounds are detected by this method which, however, does not allow a separation between this two groups.

MODIFICATIONS OF THE METHOD

From studies with a neutrophil elastase inhibitor Janusz and Durham (1997) concluded that of the destruction of the joints in rat collagen-induced arthritis is at least partially due to neutrophil elastase.

Romas et al. (2002) reported that osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis in Dark Aguti rats.

Studies in Mice

Hom et al. (1988), Takagishi et al. (1986, 1992), Cannon et al. (1990), Nemoto et al. (1992) and Carlson et al. (1992) described the effects of immunomodulating agents in collagen-induced arthritis in mice.

Wooley et al. (1993) investigated the anti-arthritic effect of recombinant human interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice.

Joosten et al. (1994) found an accelerated onset of collagen-induced arthritis in DBA₁ lac/J mice by remote inflammation.

Miesel et al. (1993, 1994a, b) studied the effects of an active center analogue of Cu₂Zn₂-superoxide dismutase in collagen type II-induced arthritis. Furthermore, the authors described a model potassium peroxochromate-induced inflammation in rats and mice. One to 3 µmol/kg K₃CrO₈ was administered by intraplantar application into the left hind-paws of anesthetized rats or mice. Arthritis index was assessed by a score system or the inflammatory response was quantified scintigraphically under a gamma camera by intravenous injection of 500 µCi Na^{99m}TcO₄.

Kumar et al. (1997) compared the cellular mechanisms involved in the control of collagen II-induced arthritis and experimental autoimmune encephalomyelitis in mice.

Ruchatz et al. (1998) studied the role of IL-15 in development of antigen-induced immunopathology in collagen-induced arthritis in DBA/1 mice. A soluble fragment of IL-15 receptor profoundly suppressed the symptoms of collagen-induced arthritis.

Joosten et al. (1999) immunized male DBA-1 mice with 100 µg bovine type II collagen in CFA enriched with *Mycobacterium tuberculosis* H37Ra (4 mg/ml) at the base of the tail. The mice were boosted i.p. with 100 µg collagen dissolved in saline. After disease onset on day 28, the mice were treated either with dimerically linked PEGylated soluble p55 TNFR1 receptor or with purified rabbit anti-murine IL-1α and anti IL-1β. IL-1αβ blockade prevented cartilage and bone destruction, whereas TNF-α blockade only ameliorated joint inflammation.

Using a similar protocol, Plater-Zyberg et al. (2001) found a therapeutic effect of neutralizing endogenous IL-18 activity in the collagen-induced model of arthritis and Lubberts et al. (2004) after treatment with a neutralizing anti-murine interleukin-17 antibody.

Cuzzocrea et al. (2003) found a reduction in the evolution of murine type II collagen-induced arthritis by treatment with rosiglitazone, a ligand of PPAR γ .

McIntyre et al. (2003) reported that a highly selective inhibitor of I κ B kinase blocked both inflammation and destruction in collagen-induced arthritis in mice.

Chen et al. (2003) tested orally active inhibitors of TNF synthesis as anti-rheumatoid arthritis drugs using collagen-induced arthritis in male DBA/1J mice.

Nakae et al. (2002, 2003) generated IL-17-deficient mice and found a suppression of collagen-induced arthritis.

Podolin et al. (2005) described attenuation of murine collagen-induced arthritis by a selective small molecule inhibitor of I κ B kinase 2, occurring via reduction of proinflammatory cytokines and antigen-induced T cell proliferation.

Kuno et al. (2006) reported anti-inflammatory activity of a non-nucleoside adenosine deaminase inhibitor in mice.

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- with the antigen in incomplete Freund's adjuvant after one and three weeks. All BALB/c mice immunized with human articular cartilage proteoglycan develop arthritis in diarthrodial joints after the third antigen injection. Sera from mice with progressive polyarthritis are tested for antibodies to arthritogenic proteoglycans during weeks 12–18 of immunization. The limbs of all mice are examined daily to record clinical arthritic changes. Swelling and redness, as the first symptoms of arthritis, and the thickness (diameter) of the knee, ankle (intermalleolar diameter), wrist and the dorso-volar thickness of the paw are recorded three times a week. The most objective joint diameter is the intermalleolar one. The animals are treated with test drug or vehicle for 12 weeks and serum samples taken by retroorbital puncture for determination of antibodies to proteoglycans. Seven weeks later, the mice are sacrificed, limbs, tails and lumbar spine are fixed, decalcified and embedded in paraffin for histological examination.

EVALUATION

Mean values of intermalleolar diameter and antibody titers of treated and non-treated animals are compared by non-parametric statistics.

MODIFICATIONS OF THE METHOD

Stimpson and Schwab (1989) described a chronic remittent erosive arthritis in rats induced by bacterial peptidoglycan-polysaccharide structures.

REFERENCES AND FURTHER READING

1.2.2.10

Proteoglycan-Induced Progressive Polyarthritis in Mice

PURPOSE AND RATIONALE

Glant et al. (1987, 1992), Mikecz et al. (1987, 1990), Poole (1989) described a proteoglycan-induced progressive arthritis and spondylitis in BALB/c mice as an animal model displaying similarities to human rheumatoid arthritis and ankylosing spondylitis as indicated by clinical assessments, immunological parameters and histopathological studies of diarthrodial joints and spine.

PROCEDURE

High buoyant density cartilage proteoglycans are prepared from fetal and adult human, canine or bovine articular cartilages as well as from 1-week-old mouse epiphyseal cartilage. Fetal human articular cartilage proteoglycan digested with chondroitinase ABC (Hascall and Heinegård 1974) is used to induce arthritis in female BALB/c mice. The mice are sensitized by intraperitoneal injection of 100 μ g of chondroitinase ABC-treated proteoglycan in 100 μ l of phosphate buffered saline, pH 7.2, and in Freund's complete adjuvant in a 1:1 emulsion. They are re-injected twice more

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I.2.2.11

Pristane-Induced Arthritis in Mice

PURPOSE AND RATIONALE

The mineral oil 2,6,10,14-tetramethylpentadecne (known as **pristane**) induces a chronic inflammatory arthritis in **mice** after intraperitoneal injection (Potter and Wax 1981; Hopkins et al. 1984; Wooley et al. 1989; Chapdelaine et al. 1991; Wooley and Whalen 1991; Levitt et al. 1992; Abe et al. 1995; Thompson et al. 1998; Wooley et al. 1998; Vigar et al. 2000). The immunological involvement in the pathogenesis of pristane-induced arthritis was studied by several authors (Bedwell et al. 1987; Thompson et al. 1990; Ghoraishian et al. 1993; Nishikaku et al. 1994; Vingsbo et al. 1996; Stasiuk et al. 1997; Morgan et al. 2004). Moreover, the genetic basis for the susceptibility to pristane-induced arthritis was studied (Lu et al. 2002; Olofsson et al. 2003; Brenner et al. 2005; Jensen et al. 2006). Not only in mice, but also in **rats**, arthritis could be induced by pristane injections (Vingsbo et al. 1996; Zheng et al. 2002, 2003; Wester et al. 2003; Holmberg et al. 2006).

Patten et al. (2004) characterized the model of pristane-induced arthritis (PIA) in mice by studying the response to antirheumatic agents, expression of joint cytokines, and immunopathology.

PROCEDURE

Induction and Characterization of PIA

Male DBA/101aHsd mice were placed under isoflurane anesthesia and injected intraperitoneally with 0.5 ml of pristane (Sigma-Aldrich, Poole, UK), and an identical booster injection was given 7 weeks thereafter. The severity of arthritis was graded visually by assessing the level of swelling in each paw, including the tarsus (ankle) or carpus (wrist) joints. The following scoring system was used: 0.5 = swelling of toes only or very slight ankle/wrist swelling; 1 = slight swelling of paw; 2 = moderate swelling of paw; 3 = marked swelling of paw; and 4 = substantial swelling of paw. Thus, the maximum total score per animal was 16. All batches also contained animals that were not treated with pristane, and these served as comparators for all studies undertaken.

Mice were observed for paw or toe swelling in a time-course study lasting up to 180 days after the first pristane injection. After study termination, the initially

swollen hind paws were obtained for histologic assessment and allocated to different study groups according to the duration of swelling. The remaining three paws of each animal were used in cytokine studies.

Drug preparation and Administration Schedules

The effects of administration of established and novel antirheumatic compounds were assessed using a therapeutic dosing schedule. Separate batches of mice for each drug study were monitored weekly for the development of swollen paws from day 80 after the first injection of pristane. Mice were included in the drug studies only if they developed a score of ≥ 1 in a hind paw on two consecutive weekly observations between day 120 and day 134 after the first injection of pristane ($n = 7-13$ per treatment group). At study termination, paws were obtained for histologic and cytokine assessments, normally at 1 h after the final drug administration.

All orally administered treatments were undertaken by gavage. Prednisolone was suspended in 0.5% methylcellulose and administered orally once daily at a dose of 2 mg/kg. Methotrexate was dissolved in physiologic saline and administered intraperitoneally 3 times per week at a dose of 9 mg/kg. Indomethacin and diclofenac were suspended in 1% methylcellulose and given orally once daily at doses of 3 mg/kg and 2 mg/kg, respectively. Celecoxib was suspended in a solution of 66% polyethylene glycol, 33% water, and 1% dimethylsulfoxide, and was administered orally twice daily at a dose of 30 mg/kg. Etanercept was dissolved in the supplied vehicle according to the instructions of the manufacturer and diluted using physiologic saline, and was administered intraperitoneally 3 times per week at doses of 300 μ g and 100 μ g per mouse. Murine sTNFR, consisting of two murine p75 receptors fused to murine IgG2a, was dissolved in physiologic saline and administered intraperitoneally 3 times per week at doses of 300 μ g and 100 μ g per mouse. The selective p38 MAPK inhibitor SB242235 (synthesized at the US GSK Research Center) was suspended in 0.5% tragacanth and 0.03M hydrochloric acid and given orally twice daily at doses of 30 mg/kg and 15 mg/kg.

Joint Cytokine Messenger RNA (mRNA) and Protein Assays

The levels of mRNA and protein for the pro-inflammatory cytokines TNF α , IL-1 β , and IL-6 were measured in disaggregated joints by TaqMan real-time reverse transcription-polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISAs),

respectively. At study termination and, in the drug studies, 1 h after the final drug treatment administration, the primary ankle joint was removed for histology and the remaining paws were removed and snap-frozen in liquid nitrogen (six to eight mice per group). For cytokine assessment, the paw showing the highest score for swelling was selected with the proviso that it had also been swollen at the start of the drug study. If the remaining three paws exhibited no swelling at study termination, then the remaining ankle was selected for assay. Whole paws were frozen and pulverized using a mortar and pestle filled with liquid nitrogen.

For the mRNA studies, total RNA was isolated from homogenized paws using RNeasy Mini-kits (Qiagen, Crawley, UK). Samples were treated with 10 units of RNase-free DNase (Qiagen) for 15 min during the RNA isolation process. Reverse transcription of mRNA was carried out using TaqMan reverse transcription reagents in an MJ Research PTC-200 PCR Peltier Thermal Cycler. TaqMan probes and forward and reverse primers for the genes of interest (TNF α , IL-1 β , and IL-6) and for housekeeping genes (GAPDH and cyclophilin) were designed with Primer Express TM software (PE Applied Biosystems). Cytokine mRNA expression levels were quantified by TaqMan real-time PCR using the ABI Prism 7900 Sequence Detector System (PE Applied Biosystems).

Measurement of Serum Antibody Levels

Blood was withdrawn from all mice before pristane injection and monthly thereafter. Levels of antibodies were determined by ELISA. Plates were coated with 100 μ l of coating buffer (0.4 M phosphate buffer, pH 7.6) containing 5 μ g of each antigen, at 4°C overnight. The antigens assessed were bovine aggrecan, bovine biglycan, human endoplasmic reticulum molecular chaperone protein, bovine chondroitin sulfate A, bovine chondroitin sulfate B, bovine type I collagen, chick type II collagen, murine type II collagen peptide, bovine decorin, bovine double-stranded DNA, human fibronectin, lupine glucose-6-phosphate isomerase, mycobacterial 65-kDa heat-shock protein, murine aggregated IgG, joint extract from normal mice, and joint extract from arthritic mice. Plates were washed 3 times with 0.05% Tween 20 in PBS and non-specific binding was blocked by 5% non-fat milk in PBS overnight at 4°C. Serum samples from at least six individual mice per time point were used. Since 1:100 was the dilution determined to produce the optimal response to high-density proteoglycans, mouse serum diluted 1:100 in 5% milk/PBS was added to each well and incubated overnight at 4°C. Subsequently,

the plates were washed 6 times with 0.05% Tween 20 in PBS and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Ala., USA) at 37°C for 1 h. Plates were again washed 6 times and developed for 40 min in the dark, using *p*-nitrophenyl phosphate as a chromagen substrate. The optical density was measured at 405 nm (OD_{405 nm}) using an ultraviolet max spectrophotometer (Molecular Devices, Sunnyvale, Calif., USA). To ensure uniformity of the assay, negative control sera obtained prior to blood withdrawal and a standard mouse anti-type II collagen antiserum were titered on each plate. Antibody binding was expressed as the OD_{405 nm} in units-blank.

Isolation of Splenocytes and Cell Proliferation Assays.

Spleens were excised and immediately immersed in PBS. Tissue was mechanically disrupted to release cells, which were suspended in 10 ml of sterile PBS and centrifuged for 10 min at 1,500 rpm. Prior to resuspension in medium, red blood cells were removed from the spleen preparations by adding distilled water for 10 s and then adding PBS. Spleen cells were then counted using a hemocytometer, and washed and resuspended in RPMI at a final concentration of 2.5×10^6 /ml.

Next, 100 μ l of spleen cell aliquots (2.5×10^6 /ml) were transferred to 96-well plates with 50 μ g/ml of each antigen (aggrecan, biglycan, chondroitin sulfate A, chondroitin sulfate B, type I collagen, type II collagen, type II collagen peptide, decorin, fibronectin, and heat-shock protein; all were derived from the same species as described for the serum antibody studies) in complete RPMI 1640 medium. Cells were incubated for 72 h at 37°C in the presence of antigen. Then 20 μ l of MTT solution (a mitochondrial enzyme substrate) was added to each well (5 mg/ml). After a 6-h incubation, the culture supernatant was discarded and 200 μ l of 10% sodium dodecyl sulfate solution was added to each well. After incubation at 37°C overnight, the OD_{590 nm} was read by microplate photospectrometer (Molecular Devices). The mean OD values were recorded for each cell sample as a measure of antigen stimulation. Antigen-specific responses were calculated as follows: (OD_{590 nm} [stimulated culture]) – (OD_{590 nm} [spontaneous proliferation culture]).

Histopathologic Evaluation

In all studies, the primary ankle joint that was swollen at the beginning of the time-course study or drug study was excised and fixed in 10% neutral buffered formalin. The tissues were decalcified with formic acid and

embedded in paraffin blocks. Sections (4–7 µm) were cut along a longitudinal axis, mounted, and stained with hematoxylin and eosin or toluidine blue, and representative slides for each animal were assessed. The following features were scored in six to ten animals per group: inflammatory exudate, neutrophil and mononuclear cell infiltration, bone resorption, and synovial hyperplasia. For drug studies, the effects of the agents on the pristane-induced pathologic condition were scored as follows: +=mild inhibition of pathologic features, ++=moderate inhibition of pathologic features, +++=marked inhibition of pathologic features.

EVALUATION

Graphic and tabular data are expressed as the mean ± SEM. Statistical significance was tested by application of the Kruskal-Wallis test for clinical scores, and by analysis of variance followed by Dunnett's test for the cytokine mRNA and protein time-course results. Antibody and cell proliferation studies were analyzed using the least-squares significant difference post hoc test.

MODIFICATIONS OF THE METHOD

Brenner et al. (2006) published thermal signature analysis as a novel method for evaluating inflammatory arthritis activity using rats with Freund's adjuvant-induced monoarthritis and pristane-induced arthritis. The thermal imaging system employs a platinum sili-cide 256 × 256 pixel detector array filtered to be sensitive to infrared radiation at a wavelength of 3–5 µm.

Lange et al. (2005) investigated the mode of action of methotrexate in different models for rheumatic arthritis, such as fibroblast-induced arthritis in SCID mice, collagen-induced arthritis and anticollagen-II-antibody-induced arthritis in rats, pristane-induced arthritis in DA rats, and models of multiple sclerosis, such as experimental autoimmune encephalomyelitis in (Balb/c x B10.Q)F1 and B10.Q mice.

Pristane induces lupus-like kidney and pulmonary disease in mice (Satoh et al. 1995; Richards et al. 1998; Lin et al. 2004; Chae et al. 2006).

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1.2.2.12

Experimental Autoimmune Thyroiditis

PURPOSE AND RATIONALE

Immunization of rats or mice with porcine thyroglobulin results in thyroiditis (Vladutiu 1971, 1983; McGregor et al. 1983; Hassman et al. 1985; Salamero et al. 1987; Fournier et al. 1990).

PROCEDURE

Crude porcine thyroglobulin (PTg) solution is emulsified in complete Freund's adjuvant in a 1:1 ratio. Female mice (6–8 weeks old) are primed with 50 μ g PTg given s.c. into four or five sites of injection and are boosted 14 days later with the same dose of PTg (s.c.) emulsified in incomplete Freund's adjuvant. The test compounds are administered from day 0 (at priming) until day 21. Mice are bled on day 21 and on day 28 after priming. The sera are tested for the levels of anti-PTg antibodies using an enzyme-linked immunosorbent assay (ELISA). On day 28, the animals are sacrificed and the thyroid glands prepared. Five-micrometer thick sections are stained with Masson-Goldner's trichrome solution.

EVALUATION

The histological severity of experimental autoimmune thyroiditis is graded as a function of mononuclear cell thyroid infiltration indices:

1. interstitial accumulation of inflammatory cells distributed between two or more follicles.
2. one or two foci of inflammatory cells reaching at least the size of one follicle
3. 10 to 40% of the thyroid replaced by inflammatory cells.
4. more than 40% of the thyroid replaced by inflammatory cells.

Mean values of treated animals are compared with controls.

MODIFICATIONS OF THE METHOD

Castagliola et al. (1994) induced autoimmune thyroid disease in BALB/c mice by immunizing with the extracellular domain of the human TSH receptor expressed as a maltose-binding protein fusion in bacteria. This type of thyroiditis could be transferred to naive BALB/c and NOD mice (Castagliola et al. 1996).

Green et al. (1995) described a spontaneous model of autoimmune thyroiditis in MRL-lpr/lpr mice.

Furthermore, Green et al. (1996) induced thyroiditis in Lewis rats by immunization with thyroid extract and thyroglobulin. A reduction of the gap junction proteins connexin 43, connexin 32 and connexin 26 was found in diseased thyroid tissue.

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I.2.2.13

Coxsackievirus B3-Induced Myocarditis

PURPOSE AND RATIONALE

The effects of immunosuppressant drugs can be studied in the murine model of Coxsackie virus B-3 myocarditis.

PROCEDURE

Three week old male BALB/c mice are kept for 7 days before the experiment in a single, self-contained animal isolation unit to exclude pre-diseased animals. They are maintained in disposable, filter-topped cages and handled with gloves by gowned and masked personnel. The intraperitoneal route is used for injection of virus in a 0.5 ml volume.

The CVB3 virus strain is grown on either Hep-2 or VERO cells, aliquotted, and maintained at -70°C until use. At the time of infection, seed virus is grown on either VERO or LLC-MK-2 cells with Dulbecco's modified Eagle medium, 12% fetal calf serum and gentamycin. Virus is harvested and adjusted to an inoculum of 1.75×10^7 plaque forming units/0.5 ml RPM-

1640. The test drugs are given subcutaneously daily for 8 days. On day 8, the animals are sacrificed, the hearts rapidly removed and divided into two equal cross sections. The basal portion is snap frozen for isolation of virus and determination of drug level. The apical portion is fixed in 10% formalin, dehydrated and embedded in paraffin. Five mm sections are stained with hematoxylin-eosin and Masson's trichrome stains. The bases of the individual hearts are minced with a sterile scalpel, suspended in 1 ml RPMI-1640, and homogenized in a glass tissue grinder. The suspension is centrifuged at 8000 g for 10 min at 4°C . Supernatants are harvested and frozen at -70°C until assay. Serial 10-fold dilutions of heart homogenates in minimum essential medium are layered on confluent, 72-h-old VERO cells, that had been grown in 96-well microtiter plates. Monolayers are checked daily for 7 days for presence or absence of virus and rate of cell destruction.

EVALUATION

The slides are examined by two observers blinded to the slide code, and inflammation and necrosis are quantitated.

MODIFICATIONS OF THE METHOD

Lane et al. (1991) showed that lipopolysaccharides promote CB3-induced myocarditis in otherwise resistant B10.A mice.

Beisel et al. (1991) identified a putative shared epitope between Coxsackie virus B4 and mouse alpha cardiac myosin heavy chain.

Gauntt et al. (1993) found that epitopes shared between Coxsackie virus B3 and normal heart tissue contribute to CVB3-induced myocarditis in mice.

Instead of Coxsackie virus B-3, Monrad et al. (1986) used encephalomyocarditis virus to induce experimental myocarditis in mice.

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1.2.2.14

Porcine Cardiac Myosin-Induced Autoimmune Myocarditis in Rats

PURPOSE AND RATIONALE

Pummerer et al. (1991), Inomata et al. (1995), Suzuki 1995; Dimitrijevic et al. (1998) described autoimmune myocarditis in rats induced by porcine cardiac myosin.

PROCEDURE

Male Sprague Dawley or Lewis rats at the age of 8 to 10 weeks are immunized with porcine cardiac myosin either purchased from Sigma (St. Louis, MO, USA) or prepared from the ventricular muscle of porcine hearts according to Murakami et al. (1976). The cardiac myosin fraction is dissolved in phosphate buffer at a concentration of 10 mg/ml. The antigen solution is emulsified with equal volume of complete Freund's adjuvant supplemented with heat-killed mycobacterium tuberculosis. Rats are injected subcutaneously into the foot pad with an immunizing dose of 5 mg of antigen in complete Freund's adjuvant/kg of body weight. Rats are injected intraperitoneally with test compounds either from day 0 to 6 (early treatment group) or from day 14 to 20 (late treatment group).

Immunized rats are sacrificed on days 8, 16, 21, and 34, respectively. Disease course and severity are analyzed by macroscopic findings of the hearts, heart weight/bodyweight ratio, as well as by histological and immunohistochemical analysis. Macroscopic findings are scored as follows: 0: normal finding; 1: presence of focal discolored area on the surface; 2: presence of diffuse discolored areas (Kodama et al. 1995).

The hearts are removed and weighted immediately after the rats are sacrificed, fixed in 10% buffered formalin and embedded in paraffin. Serial section (5 μ m in thickness) are stained with hematoxylin-eosin. The severity of myocarditis is determined according to the following scoring system: 0, no inflammation; 1: histological cross section infiltrated up to 5%; 2: 5–10% infiltrates/section; 3: 10–20% infiltrates/section; more than 20% infiltrates/section.

For immunohistochemical staining, heart samples are embedded in OCT compound (Miles, Elkhart, IN) and rapidly frozen. Cryostat sections are cut sequentially at 7 μ m in thickness, mounted on glass slides and prepared for immunoperoxidase staining. Sections are fixed in cold acetone for 10 min and extensively washed in 0.1 M Tris buffer solution, pH 7.6. Murine monoclonal antibodies specific for different rat molecules are added at appropriate concentrations. After incubation at 4°C over night and further buffer washes, the sections are incubated with peroxidase-conjugated anti-mouse immunoglobulins for 60 min. Peroxidase reaction is visualized with 0.05% diaminobenzidine in 0.01% H₂O₂ for 7–8 min. The color development is stopped by washing slides in running water. All samples are lightly counterstained with hematoxylin, mounted in gelatin/glycerol medium and assessed by light microscopy.

EVALUATION

Macroscopic and microscopic scores are expressed as mean values. Body weights, heart weights and heart weight/body weight ratio are expressed as mean \pm SD. Student's *t*-test for paired samples is used for comparison data within groups in reference to time, while two-sample *t*-test is used for comparison data between groups.

MODIFICATIONS OF THE METHOD

Koyama et al. (1995) immunized Lewis rats with human cardiac myosin suspended in complete Freund's adjuvant and induced severe active myocarditis with acute and chronic heart failure. The baseline left ventricular pressure was significantly lower in the chronic phase group and peak *dP/dt* was significantly lower in both the acute phase group and the chronic phase group than in the respective controls. The animal model was recommended to study both acute heart failure related to acute myocarditis and chronic heart failure due to diffuse myocardial fibrosis.

Neu et al. (1990, 1991a,b; Penninger et al. 1993) induced severe autoimmune myocarditis in some mouse

strains by immunization with cardiac myosin in complete Freund's adjuvant.

Wahed et al. (2005) used the method of immunization with porcine cardiac myosin to test the effects of eplerenone, a selective aldosterone blocker, on the progression of left ventricular dysfunction and remodeling in rats with dilated cardiomyopathy.

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This pathological model is an immunologic disease arising from a delayed hypersensitivity reaction to nervous tissue. In many respects, the model resembles autoimmune diseases, especially demyelinating diseases, in man. The method is used for evaluation of immunosuppressive properties of drugs.

PROCEDURE

Preparation of the encephalitogen: 3 g of spinal cord from guinea pigs or rats are homogenized with 7.5 ml bidistilled water, 3.8 ml phenol and 7.5 ml complete Freund's adjuvant under cooling.

Groups of 6–12 male Wistar–Lewis rats with an initial body weight of 130–200 g are used. On day 0, experimental allergic encephalomyelitis is induced by subplantar injection of 0.1 ml of the encephalitogen into the left hind paw. An equal volume of *Bordetella pertussis* vaccine concentrate (200×10^9 organisms/ml) is injected into the same foot. From days 1–2, the animals receive the test compound or vehicle only or the standard drug by oral administration once a day. Body weights of the animals are recorded every second day. The clinical signs of experimental allergic encephalomyelitis consist of ataxia or paresis, i.e., grossly irregular gait and weakness of one or both hind legs followed by flaccid paralysis of the hindquarters, urinary incontinence, fecal impaction and abdominal wall flaccidity. Animals showing one of these clinical signs are considered positive for the purpose of evaluation.

EVALUATION

Starting from day 7, the severity of clinical signs and mortality are determined daily and scored according to the following scheme:

| | score |
|---------------------------|-------|
| per 20 g loss of weight | 1 |
| paralysis of the tail | 1 |
| paralysis of the hind paw | 3 |
| complete paralysis | 5 |
| death | 6 |

Calculation of the Results

The delay of onset of the paralytic symptoms is determined. The total score per day is recorded for treated and control groups. On the day of maximal clinical symptoms occurring among control animals, the total scores of the treated groups is compared to the total score of the control group. The percentage change is evaluated.

I.2.2.15

Experimental Allergic Encephalomyelitis

PURPOSE AND RATIONALE

Experimental allergic encephalomyelitis was first produced in laboratory animals by Rivers et al. in 1933.

Doses of 0.5 mg/kg p.o. methotrexate, 1 mg/kg p.o. hydrocortisone, 2.5 mg/kg p.o. cyclophosphamide were found to be active, whereas non-steroidal anti-inflammatory compounds were inactive.

CRITICAL ASSESSMENT OF THE METHOD

The model of experimental allergic encephalomyelitis in rats is suitable to distinguish between immunosuppressive and anti-inflammatory drugs. Experimental autoimmune encephalomyelitis is considered as a rodent model of the autoimmune disease multiple sclerosis (Pearson et al. 1997; Deng et al. 2002).

MODIFICATIONS OF THE METHOD

The phosphodiesterase inhibitor pentoxifylline was found to prevent induction of experimental autoimmune encephalomyelitis in Lewis rats (Rott et al. 1993).

Martin and Near (1995) studied the protective effect of the interleukin-1 antagonist IL-1ra on experimental allergic encephalomyelitis in Lewis rats.

Experimental autoimmune encephalomyelitis in different strains of mice was described by Heremans et al. (1996), Glabinski et al. (1997), Liblau et al. (1997).

Baker et al. (1990, 1991, 2000) induced experimental allergic encephalomyelitis in Biozzi AB/H mice by sensitization with 1 mg of mouse spinal cord homogenate emulsified in Freund's complete adjuvant on days 0 and 7. The disease is characterized by relapsing-remitting episodes similar to multiple sclerosis in human beings. Biozzi AB/H mice also develop spasticity and tremor which can be antagonized by cannabinoids.

A chronic relapsing-remitting form of experimental autoimmune encephalomyelitis was induced in the common marmoset *Callithrix jacchus* following a single immunization with human white matter by Massacesi et al. (1995), Genain and Hauser (1997) and recommended as a new model for multiple sclerosis. This model has been used for histopathological characterization of magnetic resonance imaging-detectable white matter lesions in a primate model of multiple sclerosis by 't Hart et al. (1998, 2004) (see E.10.3.2).

Experimental allergic neuritis in several animal species has been described by Waksman and Adams (1955, 1956; King et al. 1983; McCombe et al. 1990; Nakayasu et al. 1990). This disorder has been considered to show similarities to the Guillain-Barré syndrome in man. The demyelating process initiated by the injected antigens is a lymphocyte-mediated reac-

tion in which activated macrophages strip myelin off the axons. Hartung et al. (1987) described the adoptive transfer experimental autoimmune neuritis in Lewis rats by injection of P2-reactive T lymphocyte cell lines.

Mix et al. (1992) studied the effect of stilbene-type anion channel blockers on the immune response during experimental allergic neuritis induced by bovine peripheral myelin.

Kojima et al. (1994) investigated the pathogenic potential of autoimmune T cell responses to nonmyelin autoantigens in the Lewis rat using the astrocyte-derived calcium-binding protein S100 β , as a model non-myelin autoantigen. In contrast to the experimental autoimmune encephalomyelitis induced by the adoptive transfer of myelin basic protein-specific T line cells, S100 β -specific T cell transfer induced intense inflammation not only in the spinal cord but also throughout the entire CNS and also in the uvea and retina of the eye.

Gautam et al. (1992) reported that a polyalanine peptide with only five native basic protein residues induces autoimmune encephalomyelitis in mice. This peptide, called myelin basic protein (MBP) Ac1-11, has been used by several authors for further studies on experimental autoimmune encephalomyelitis (Ratts et al. 1999; Matejuk et al. 2003).

Pearson et al. (1997) reported the induction of a heterogeneous T-cell receptor repertoire in (PL/JXSJL/J)F2 mice by myelin basic protein peptide Ac1-11 and its analog Ac1-11[4A].

Deng et al. (2002) found that expression of the tyrosine phosphatase Src homology 2 domain-containing protein tyrosine phosphatase 1 determines the T cell activation threshold and severity of experimental autoimmune encephalomyelitis.

Maron et al. (2002) investigated the immunological properties of Cop1 (glatiramer acetate) to determine the degree to which its effects were antigen specific using myelin basic protein T-cell receptor transgenic mice. Immunization of these mice fed glatiramer acetate, myelin basic protein or MBP Ac1-11 resulted in decreased proliferation, and IL-2, IL-6, and IFN- γ production, and increased secretion of IL-10 and TGF- β in glatiramer acetate-fed animals.

Gilgun-Sherki et al. (2003) reported that riluzole suppresses myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in mice.

Pollak et al. (2003) studied the experimental allergic encephalitis-associated behavioral syndrome and the modulation by anti-inflammatory treatments.

Diab et al. (2004) found that ligands for the PPAR- γ and the retinoid X receptor exert additive anti-inflammatory effects on experimental autoimmune encephalomyelitis. Duckers et al. (1997) studied the effect of a neurotropic treatment on cortical lesion development in experimental allergic encephalomyelitis in rats by longitudinal *in vivo* magnetic resonance imaging methods.

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1.2.2.16

Acute Graft Versus Host Disease (GVHD) in Rats

PURPOSE AND RATIONALE

The intravenous injection of a mixture of parental splenocytes into healthy inbred F₁-rats results in graft-versus-host (GVH) induced immune abnormalities. This is due to T-lymphocytes in the donor inoculum that recognize the major histocompatibility alloantigens expressed by the F₁-animals. The host F₁ T-cells are genetically unable to recognize antigens of the parental donor as foreign, thus the response involves only donor recognition of host and not host recognition of donor. The ensuing immune abnormalities lead to clinical symptoms of an acute, lethal GVH-disease (GVHD), i. e. profound immunodeficiency, anemia, hypogamma-globulinemia and runting.

PROCEDURE

Three to 4 month old, male F₁-hybrid rats of the inbred strains Lewis (Rt-1 l) and Brown Norway (BN, Rt-1n) (Zentralinstitut für Versuchstierkunde Hannover Germany) are used as hosts for cell grafts from the Lewis parental strain. The bone marrow cells are obtained by flushing hind femur bone shafts with culture medium. These cells are then pooled together with spleen cells (ratio 2 bones: 1 spleen). The cell viability, determined by trypan exclusion, has to be more than 90%. Each recipient is injected with about 40 × 10⁷ cells in a 1.5 ml-suspension volume. The route of injection is the penis vein, allowing an optimal control of correct intravenous application.

Prophylactic Drug Application

For this experiment, 2 groups of 6 F₁-hybrids each are injected with the above mentioned bone marrow/spleen cell suspension. One group receives the test drug orally and daily until the end of the experiment, homogeneously suspended in 1% carboxymethylcellulose (CMC) solution. The other group receives CMC alone and, thus, serving as the GVHD control group. The experiment is terminated two weeks after disease induction i. e. 1 week after the first appearance of GVHD-symptoms. All animals are sacrificed and clinical aspects documented, spleens weighed, histology of the skin, liver, spleen and lymph-nodes performed and organs photographed.

Therapeutic Drug Application

In this experiment, rats are separated into four groups treatment begins with the first sign of GVHD-symptoms (beginning of the second week). Because of

the expected, greater therapeutic difficulty, the daily dose of the test drug has to be doubled, again for 2 weeks duration.

The experiment is terminated either by sacrificing those rats that are too sick to be able to move around the cage, or at the end of the 4 week observation period, regardless of the clinical condition of the animals. The clinical-chemical parameters are determined by routine procedures conducted with a HITACHI autotechnicon.

EVALUATION

The tested parameters of therapeutic success or disease, respectively, are survival rate (%), spleen weight (g), and body weight (g) as well as clinical-chemical parameters (bilirubin, alkaline phosphatase, creatinine, white cell count) after 2 and 3 weeks.

MODIFICATIONS OF THE METHOD

Gelpi et al. (1994) established a chronic graft vs host disease in (C5BL/10 x DBA/2)F₁ mice with an injection of lymphoid cells from the parent DBA/2 strain. Most of the animals developed antibodies against transfer RNA/protein particles.

Mosier et al. (1988) reported transplantation of human peripheral blood lymphocytes (PBL) into severe combined immunodeficient (scid) mice to construct hu-PBL-scid mice. Kim et al. (1997) suggested these mice for routine immunotoxicity investigations using lymph nodes of intestines as the lymphocyte sources.

Ford et al. (1970), Schorlemmer et al. (1997, 1998) used the popliteal lymph node assay to study the local graft vs. host reaction. The test is based on the enlargement of the draining popliteal lymph nodes as a result of injecting immuno-competent cells (1×10^8 parental Lewis spleen cells) into the hind foot pad of Lewis x Brown-Norway F₁ recipients. The reaction is measured at day 6 after challenge as a gain in lymph node weights.

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I.2.2.17

Influence on SLE-Like Disorder in MRL/lpr Mice

PURPOSE AND RATIONALE

Systemic lupus erythematosus (SLE) is an autoimmune disease in man that affects multiple body organs and is characterized by the development of certain types of self antigens. Primarily, the antibodies formed against double-stranded DNA (dsDNA), the most prevalent in this ailment, complex together and, with complement, deposit in the small blood vessels, leading to widespread vasculitis. MRL Mpf lpr/lpr

(MRL/lpr)-mice spontaneously develop a severe disease with many symptoms very similar to human SLE, i. e. hypergamma-globulinemia, and glomerulonephritis (Theofilopoulos and Dixon 1981).

PROCEDURE

Female MRL/lpr-mice (originally from Jackson Laboratories, USA), displaying distinct symptoms of SLE (between 12 and 13 weeks of age) are randomized and divided into groups of 12 animals each. At this age, the animals have already clinical manifestations of the SLE-like illness, as determined by the disease index, but have not yet developed proteinuria. Animals with early symptoms of disease are treated with various drugs, e. g., leflunomide, cyclosporin A, azathioprine, cyclophosphamide or prednisolone for 11 weeks and the survival rate and disease index of these animals are followed for 24 weeks. The disease index and urine protein level are determined once weekly.

Disease Index

The subsequent clinical parameters are taken into consideration:

1. Ears: reddening of the skin, deterioration of the pinna.
2. Nose: loss of hair, wasting of the skin.
3. Lymph nodes: detection of swollen lymph nodes on any part of the body, especially neck and extremities.
4. Fur: general condition of fur (e. g. shabby, mangy, etc.), loss of hair.
5. Skin: inflammation of the skin, scab and/ or granuloma formation.
6. Eyes: exophthalmos, deterioration due to inflammation, tumor formation around the eye, swelling of the eyelid with eventual closure of the eye.
7. Paws: reddening of the skin, swelling of the paw.

EVALUATION

A score for each of the above described parameters is given according to the severity of the symptoms as follows:

Points for clinical index

| Involvement | Detectable | Moderate | Severe |
|-------------------|------------|----------|--------|
| Ears (each) | 0.5 | 1.0 | 1.5 |
| Nose | 1.0 | 2.0 | 3.0 |
| Lymph node (each) | 1.0 | 2.0 | 3.0 |
| Fur | 1.0 | 2.0 | 3.0 |
| Skin | 1.0 | 2.0 | 3.0 |
| Eyes (each) | 1.0 | 2.0 | 3.0 |
| Paws (each) | 0.5 | 1.0 | 1.5 |

Body weight (one point for 5 g difference from week to week)

The determination of the disease index is performed, weekly, by the same individual, but without knowledge of the group being evaluated. The points, for each animal, are registered and the total score, of each group, summarized. The average score for the group is calculated, significance between the experimental group and the untreated diseased group is determined using the Student *t*-test.

Proteinuria

Pooled urine is collected from each experimental group and the amount of protein in the urine is calculated.

MODIFICATIONS OF THE METHOD

In addition to a lupus-like syndrome and massive T cell proliferation. MRL-1pr/1pr (MRL/1) mice develop an arthritic process very similar serologically and histologically to human rheumatoid arthritis. Boissier et al. (1989) found that in these animals mouse type II collagen is antigenic, but not arthritogenic.

Holmdahl et al. (1991) studied the involvement of macrophages and dendritic cells in synovial inflammation of collagen induced arthritis in DBA/1 mice and spontaneous arthritis in MRL/Lpr mice.

Rordorf-Adam et al. (1985) used serum amyloid P component and autoimmune parameters in the assessment of arthritis in MRL/lpr/lpr mice.

Furukawa et al. (1996) studied the autoimmune disease-prone genetic background in relation to Fas-defect in MRL/lpr mice.

Kanno et al. (1992) found spontaneous development of pancreatitis in the MRL/Mp strain of mice.

Kusakari et al. (1992) compared hearing acuity and inner ear disorders of MRL/lpr mice with those of BALB/c mice and found a significantly higher auditory brain stem response threshold. They recommended this as a model of sensorineuronal hearing loss.

Bundick and Eady (1992) investigated the effects of an immunosuppressive agent on the development of spontaneous lupus disease in female NZBW F₁ hybrid mice.

Walker et al. (1996) reported a powerful suppressive effect of testosterone on the autoimmune disease analogous to systemic lupus erythematoses spontaneously developed by F₁-hybrids of New Zealand Black (NZB) × New Zealand White (NZW) mice. A model was developed in which NZB dams carrying NZB/NZW fetuses were treated with testosterone

in a dose adequate to masculinize the external genitalia in female fetuses.

Zoja et al. (1998) investigated bindarit, a compound devoid of immunosuppressive properties, in NZB/W F1 hybrid mice developing an immune complex glomerulonephritis with proteinuria and progression to renal insufficiency.

Kiberd and Stadnyk (1995) studied the role of endogenous interleukin-1 in established lupus nephritis in MRL lpr/lpr mice by administration of the IL-1 receptor antagonist IL-1ra.

Gleichmann et al. (1982), Schorlemmer et al. (1997) induced a systemic lupus erythematoses-like disease in mice by abnormal T- and B-cell cooperation. A chronic graft versus host reaction with the pathologic symptoms of severe glomerulonephritis is induced in B6D2 (C5B1/6 × DBA/2) F1 hybrid mice receiving four i.v. injections (one per week) of 1×10^8 parental lymphoid spleen cells from DBA/2 donors. The inoculation of splenocytes into the BDF1 hybrid mice results in the development of a chronic GvH reaction with lymphoid hyperplasia, autoantibody production and immune-complex glomerulonephritis.

Chan et al. (1995) described ocular changes occurring in mice with experimental lupus erythematoses. The ocular disease is characterized by bilateral subacute and chronic inflammation of the eyelids (blepharitis) and hypertrophic Meibomian glands. The severity of the ocular changes is strain dependent. The authors recommend this experimental eye disease as an animal model for chronic blepharitis in humans.

The changes of lacrimal and salivary glands found in MRL/lpr mice and other mouse strains with autoimmune disorders were also regarded as model of Sjögren's syndrome in human (Sullivan and Edwards 1997; Toda et al. 1999).

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1.2.2.18

Prevention of Experimentally Induced Myasthenia Gravis in Rats

PURPOSE AND RATIONALE

Myasthenia gravis is an organ specific autoimmune disease in man that results in skeletal muscles weakness. Typically, the sufferer has drooping eyelids, a blank facial expression, and weak, hesitant speech. This is due to the formation of autoantibodies against the nicotinic acetylcholine receptor (AChR). The formation of autoantibodies to acetylcholine's receptor leads to a gradual destruction of the receptors in skeletal muscles that receive nerve impulses and initiate muscle contractions. As a result, affected muscles fail to respond or react only weakly to nerve signals.

Experimental myasthenia gravis (EMG) can be induced in rats by injecting them with heterologous AChR, or with recombinant α subunits (two) of the AChR (portion of the AChR to which acetylcholine mainly binds) (Lennon et al. 1991). The animals display symptoms of myasthenia (electrophysiological evidence of altered neuromuscular function) and detectable antireceptor antibodies. The two α -subunits are plausible targets of pathogenic autoantibodies because they would allow mechanisms that depend on cross-linking, complement-mediated lysis, or hindrance of acetylcholine binding to the receptor (Schwartz 1993). The severity of the disease can vary, but most animals display, at the very least, a weakness and fatigability of foot grip. The disease gradually leads to abnormal gait and eventually the inability of the animals to walk or even right themselves.

PROCEDURE

Female rats of AO strain, 6–10 weeks old, are used. Three groups of rats are included in the experiment:

1. immunized with acetylcholine receptor (AChR)-protein and treated with test drug;
2. immunized with AChR-protein without drug, and
3. nonimmunized, nontreated control rats. The test drug is applied per os daily. First dose is administered on the day of immunization and the last on the day of sacrifice.

Immunization with AChR-Protein

AChR-protein isolated from *Torpedo marmorata* is emulsified with complete Freund's adjuvant and

100 $\mu\text{g}/\text{rat}$ is injected intradermally in the hind foot pad. As additional adjuvant, 2.6×10^{10} *Bordetella pertussis* microorganisms is administered simultaneously by intramuscular injection in the hind leg.

Antibody Determination

Anti-AChR-protein antibodies are measured by enzyme linked immunosorbent assay (ELISA) as described by Norcross et al. (1980). AChR-protein is diluted to a final concentration of 2.5 $\mu\text{g}/\text{ml}$ in 0.05 M carbonate buffer, pH 9.6. Two hundred ml of this solution is placed in each well of a microtitration plate (Flow Laboratories Inc.). After an over night incubation at 4°C, the plates are washed thoroughly with 0.01 M phosphate buffered saline (PBS) solution containing 0.05% Tween-20 (Sigma) subsequently referred to as PBS/T. Sera from all groups of rats are serially diluted in PBS/T and 200 μl is added to each micron well except in the background row (control row) and incubated at 4°C for 2 h. After washing, 200 μl of 1:1000 diluted peroxidase conjugated goat antirat immunoglobulin (Sera Lab. Sussex, England) in PBS/T are added to the micron wells and incubated for an additional 60 min at 4°C. After plates are washed, 200 μl of substrate-citrate buffer and 0.2 μl of 10% H_2O_2 are added and then incubated in the dark at room temperature for 30 min. The reaction is stopped by addition of 50 μl of 2M H_2SO_4 and the OD determined by using Titert Multiscan.

Two-color Flow Cytometry

Thymic cell suspensions are obtained by mincing tissue and passing it through 80 mm stainless mesh. After being washed three times in PBS, the cells are resuspended in PBS at a cell density of 10^7 viable cells/ml. The cell viability is determined by the trypan blue exclusion test. Erythrocytes are removed by addition of ammonium chloride. Cell staining and flow cytometric analyses are done as described by Itoyama et al. (1989). Thymocyte subsets expressing CD4 and/or CD8 molecules are defined by staining with monoclonal antibodies obtained from Serotec, Oxford, England: phycoerythrin (PE)-conjugated anti-W3/25 (CD4) and fluorescein isothiocyanate (FITC)-conjugated anti-MRC OX8 (CD8). 2×10^5 to 1×10^6 cells suspended in 100 ml of PBS are exposed sequentially for 30 min to FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 monoclonal antibodies. Isotype matched control monoclonal antibodies are used to prove the specificity of binding. Cell analysis is performed using FACScan flow cytometer from Becton Dickinson. 1×10^4

events per sample are analyzed by Consort 30 and Lysis software. All data are collected and displayed on a log scale of increasing green and orange fluorescence intensity. This is presented as two-dimensional contour maps and as percentage of thymocytes by integrating counts in selected areas of the contour plots.

Stereologic Analysis of Thymuses

Thymuses of animals of all groups are prepared for light microscopic analysis. For this purpose thymus tissue is fixed in Carnoy's solution, embedded in paraffin and 3–5 µm thin sections are stained with hematoxylin and eosin. Cortex and medulla are analyzed stereologically using the point counting method described by Weible (1963). Volume density (V_v) of the examined structures is determined by the following equation: $V_v = P_i/P_t$, where P_i represents the number of points of the examined structure, and P_t the total number of points. V_v refers to the volume fraction i. e. volume of a feature per unit test volume (Williams 1981).

EVALUATION

EMG is evaluated clinically by daily examination of muscle weakness and scored as follows:

+ = weakness of grip with fatigability;

++ = abnormality of gait;

+++ = inability to walking and righting.

Immediately after appearance of clinical signs of EMG, rats are sacrificed, blood and thymuses are taken for determination of anti-AChR-protein antibodies, and histological analysis of thymuses and thymocyte subsets, respectively.

Statistical analysis of data is performed by Student's *t*-test (data of stereological analysis) and Mann-Whitney *U*-test (Results of flow cytometric analysis of thymocyte subsets).

MODIFICATIONS OF THE METHOD

McIntosh and Drachman (1987) described an *in vitro* suppressor assay using responder cells from the lymph nodes of Lewis rats immunized sc. with acetylcholine receptors emulsified in complete Freund's adjuvant and suppressor cells from spleens of rats immunized i.p. with acetylcholine receptors absorbed on bentonite. Antibodies were determined after stimulation with acetylcholine receptors from co-cultures of responder cells and putative suppressor cells treated previously with an immunosuppressant.

Arago and Blalock (1994) developed a method of altering B-cell-mediated autoimmune diseases by induction of anti-idiotypic antibodies by immunization

with complementary peptides. A peptide encoded by RNA complementary to RNA for the Torpedo acetylcholine receptor main immunogenic region, AChR 67–16, was tested in the Lewis rat model of experimental autoimmune myasthenia gravis.

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I.2.2.19

Glomerulonephritis Induced by Antibasement Membrane Antibody in Rats

PURPOSE AND RATIONALE

Masugi nephritis and other nephritis models of immunological origin in rats have been used for evaluation of immunosuppressive activity (Heymann et al. 1959; Shibata et al. 1966; Ito et al. 1983; Thoenes et al. 1989; Ogawa et al. 1990, 1991).

PROCEDURE**Preparation of Rabbit Antiserum against Rat Glomerular Basement Membrane**

Glomeruli are separated from the homogenate of rat renal cortex by successive use of 3 metal sieves (150-, 180-, and 200-mesh). The basement membrane fraction is obtained by centrifugation and ultrasonic disruption. It is then digested with trypsin, dialyzed, and lyophilized. The resultant substance is employed as antigen. An emulsion of 1 mg of the antigen in 0.2 ml saline with 0.2 ml of complete Freund's adjuvant is injected intracutaneously into white rabbits once a week for 6 weeks. One week later, production of the antibasement membrane antibody is confirmed in guinea pigs by the passive cutaneous anaphylaxis test. Blood is collected from the carotid artery, incubated at 56°C for 30 min to inactivate components of the complement, and stored at -20°C until use.

Induction of Glomerulonephritis in Rats

Male Sprague-Dawley rats weighing about 300 g are injected with 0.5 ml of the rabbit antiserum via the tail vein. On the following day, they are further injected subcutaneously with an emulsion (0.25 ml) of physiological saline solution containing 5 mg of rabbit gamma-globulin in an identical volume of complete Freund's adjuvant.

Treatment

The rat antibasement antibody is injected 5 days before the start of administration of the test compound. Before the first dose, urinary total protein is determined and rats with nephritis are so assigned as to provide almost equal distribution of severity of the disease per group. The test compounds are administered orally for 14 days. The urine is collected at 7 and 14 days of treatment. After 14 days, the animals are sacrificed, blood is collected and thymus and kidneys are removed. Histopathological and immunohistochemical studies are performed in kidney tissue.

EVALUATION

Scores are given for **microscopic findings in the glomeruli:**

- cell proliferation in glomeruli
- PAS-positive granules in the epithelium of glomeruli
- fibrin deposits in Bowman's space
- adhesion to Bowman's capsule

and in **the tubuli:**

- hyaline cast
- dilation of tubuli

as well as for **immunofluorescence findings** for rat IgG, rat C3, and rabbit IgG.

Furthermore, total urinary protein, plasma total cholesterol, plasma fibrinogen, thymus/body weight ratio are compared between drug treated animals and controls by statistical means.

MODIFICATIONS OF THE METHOD

Lan et al. (1995) investigated the pathogenic role of interleukin-1 in the progression of established rat crescentic glomerulonephritis by administration of the interleukin-1 receptor antagonist IL-1ra.

Giménez et al. (1987), Thoenes et al. (1987) induced autoimmune tubulointerstitial nephritis in the brown Norway rat by injection of bovine tubular basement membrane.

Development of a systemic T-lymphocyte dependent autoimmune syndrome in brown Norway rats including glomerulonephritis with high proteinuria was induced with mercuric chloride by Baran et al. (1986), Aten et al. (1988), Lillevang et al. (1992).

Kokui et al. (1992) induced nephrosis with proteinuria in rats by intraperitoneal injection of puromycin aminonucleoside.

Lundstrom et al. (1993) studied the Heymann nephritis antigenic complex using a rat yolk sac carcinoma cell line that expresses glycoprotein 330, the main antigen in this autoimmune disease.

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I.2.2.20

Auto-Immune Uveitis in Rats

PURPOSE AND RATIONALE

The mechanisms of certain types of uveitis have been studied in animals implicating autoimmunity. Uveitis has been produced in guinea pigs following injections with homologous uveal tissue.

The method is described in detail in the Sect. O.7.3.

I.2.2.21

Inhibition of Allogenic Transplant Rejection

PURPOSE AND RATIONALE

Transplantation of allogenic organs to recipients results in rejection of the transplants. This effect can be suppressed or delayed by immunosuppressive agents. Various organs are used for allogenic transplantation in animal experiments, such as skin pieces (Schorlemmer et al. 1993), kidney (Lee 1967; Küchle et al. 1991), rat heart, rat small intestine (Xiao et al. 1944) and corneal buttons (Coupland et al. 1994). The immunosuppressive activity can be evaluated either by using a major histocompatibility complex variant strain combination or a strong allogenic system.

PROCEDURE

For **skin transplantation** male animals of inbred strains of Fischer (F334), Lewis (LEW), Brown Norway (BN), Dark Agouti (DA) rats are used. Rat tail skin (donor) is cut into square pieces of 0.5 to 1.0 cm and transplanted to the tails of recipient rats. Rejection is defined as the day when the skin graft is of red-brown color and hard consistency. As strain combination with a major histocompatibility variant, transplantation from LEW to F334 is performed. Using a strong allogenic system, the high responder DA to LEW donor recipient combination is used. The immunosuppressive agents, e. g., cyclosporine or leflunomide are given orally up to 20 days. Ten animals are used for each group.

EVALUATION

The mean values of rejection time of treated groups are compared statistically with vehicle treated controls using Student's *t*-test or the Mann Whitney U-test.

MODIFICATIONS OF THE METHOD

Schorlemmer and Kurrle (1997) used Lewis (LEW, Rt1*) rats as receivers and Balb/c mice as donors

in a xenotransplantation model of mouse-to-rat skin grafts. Rejection was defined as the day when the skin graft turned red-brown and became hard. For quantification of xenospecific IgM and IgG antibody titers, the test sera (dilution 1:10) were incubated with 1×10^6 purified T-cells (by sheep anti-mouse dynabeads, Deutsche Dynal GmbH, Hamburg, Germany) from Balb/c donor spleens for 30 min at 4°C. The cells were washed 3 times with phosphate buffered saline (pH 7.2) and then stained for IgG or IgM xenoantibodies; 50 µl of either FITC-conjugated goat antibodies, specific for the Fc-portion of rat IgG or specific for the μ -chain of rat IgM were added. After 30 min at 4°C the cells were washed twice and analyzed by flow cytometry.

Techniques for transplantation of several organs have been elaborated.

For **kidney transplantation** male rats, 5–7 months of age, are used as donors and recipients for the orthotopic right kidney transplantation as described by Lee (1967) with a modification of ureter-ureter anastomosis (Thoenes et al. 1974). Because bilateral nephrectomy is performed at transplantation, animal survival is dependent upon the allograft's function. All rats that do not excrete urine on the first postoperative day are excluded from further studies. As a control concerning long survival, syngeneically transplanted rats are maintained up to 300 days.

Engelbrecht et al. (1992) described a new rapid technique for renal transplantation in the rat. The method combines a special sleeve anastomotic technique for the renal artery, conventional end-to-end anastomosis of the renal vein, and implantation of the ureter into the bladder.

A porcine renal transplant model has been used by Almond et al. (1992).

Peters et al. (1993) reviewed the therapeutic potential of tacrolimus in renal and hepatic transplantation.

For studying **heart transplantation**, heterotopic implantation of hearts from BN to LEW rats is performed (Williams et al. 1993). The diagnosis of rejection is established once the palpable cardiac allograft impulse ceases. Further studies with rat cardiac allografts have been performed by Hancock et al. (1990). The Fischer 344 rat (donor)/Long Evans rat (recipient) combination was used by Kahn et al. (1991). Walpoth et al. (1993) used magnetic resonance spectroscopy for assessing myocardial rejection in the transplanted rat heart.

Shiraishi et al. (1995) evaluated the effectiveness of the interleukin-1 receptor antagonist IL-1ra in the

immune and inflammatory responses to rat heart allografts.

Cardiac transplantation between inbred rat strains that differ for weak histocompatibility antigens is associated with the development of arteriosclerosis in arteries of the donor graft myocardium (Carmer et al. 1990; Adams et al. 1992).

A heterotopic rat **heart-transplant model** and the influence of infection was described by Kobayashi et al. (1993).

The **hamster to rat cardiac xenograft** model has been used by several authors (de Masi et al. 1990; Steinbrüchel et al. 1991; van den Bogaerde et al. 1991; Woo et al. 1993; Fujino et al. 1994; Schuurman et al. 1994). The hearts from Syrian hamsters were implanted heterotopically in male Lewis rats, with anastomoses between the infrarenal abdominal aorta and inferior vena cava of the recipient and the donor aorta and right pulmonary artery, respectively.

Primate cardiac xenografts were performed by McManus et al. (1993) using cynomolgus monkeys (*Macaca fascicularis*) as donors and baboons (*Papio anubis*) as recipients.

Chronic rejection of rat **aortic allograft** was studied by Mennander et al. (1991). Administration of cyclosporin induced accelerated allograft arteriosclerosis.

Heterotopic transplantation of small intestine has been performed from BN to LEW rats. The mesenteric venous drainage is reconstructed either via the vena cava or the portal vein (Xiao et al. 1994). An isolated Thiry–Vella-loop was prepared by Xia and Kirkman (1990). Kellnar et al. (1990) described allogenic transplantation of fetal rat intestine with anastomosis to the normal bowel of the host. Langrehr et al. (1991) investigated under which circumstances graft versus host disease occurs following fully allogenic small bowel transplantation in the rat. Kirsch et al. (1991) studied the extent to which intestinal transplants in rats undergo functional and morphologic compensation.

Liver transplantation procedure has been described by Svensson et al. (1995) allowing measurement of bile secretion.

Orthotopic left **lung transplantation** was performed in inbred rats by Katayama et al. (1991).

Tracheal allografts were implanted into the abdomen of recipient rats (Davreux et al. 1993).

In vivo electrophysiology of rat **peripheral nerve transplants** was studied by Yu et al. (1990). A sciatic-tibial nerve graft was harvested from the donor rat between the sciatic notch and the ankle. In the recipient, the tibial nerve and the sural nerve were resected. The

nerve graft was placed along the natural course of the native tibial nerve. Nerve repair was performed using standard end-to-end epineural microsuture technique.

A model of neurovascularized rectus femoris **muscle transplantation** in rats was established by Muramatsu et al. (1994).

The orthotopic **transplantation of vascularized skeletal allografts** (rat distal femur and surrounding muscular cuff) has been described by Lee et al. (1995).

Long-term surviving of **limb allografts** in rats was studied by Kuroki et al. (1991). The donor and recipient limbs were prepared simultaneously by amputation at midfemur. The donor limb was fixed orthotopically by Kirschner wire. The donor and recipient femoral arteries, veins and sciatic nerves were anastomosed using a microsurgical technique.

For **cornea transplantation** Brown Norway rats (RT1^{Lxn}) serve as donors and Lewis rats (RT1^l) as recipients (Coupland et al. 1994). Both the donor and recipient rats are anesthetized with xylazine hydrochloride and ketamine hydrochloride. Twenty min prior to surgery the recipient rats also receive 0.5 mg/kg atropine sc. and phenylephrine hydrochloride 5% eye-drops. Under sterile conditions and using an operation microscope, two donor corneal buttons (3.5 mm) are harvested from the donor rat using a trephine and curved Castroviejo scissors. The donor animals are then sacrificed by ether inhalation. The left eyes of the recipient rats are prepared by removing a central 3.0-mm button using a trephine and curved Castroviejo scissors. A drop of sterile methylcellulose (1%) is placed over the 3.0-mm corneal opening before the donor cornea is fixed with 10 interrupted sutures. The anterior chamber is not re-established following surgery. Prior to closure of the eyelids with 3 or 4 interrupted sutures, Polyspectran eyelid gel is placed over the operated eye. Forty-eight hours following surgery, the eyelid sutures are removed, allowing for the first time assessment of the cornea on the slit-lamp microscope. Slit-lamp evaluations are performed every 2–3 days under i.m. anesthesia with ketamine, with assessment of the cornea by scoring graft opacity, edema and vascularization.

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Chapter J

Activity on the Gastrointestinal Tract¹

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J.1 Salivary Glands

J.1.0.1

Measurement of Salivation

PURPOSE AND RATIONALE

Symptoms of several human diseases are manifested as increased salivation (e. g., Parkinson's disease) or decreased salivation (e. g., xerosis). Studies to find and to evaluate sialagogues, such as substance P and its synthetic derivatives, as well as to search for salivation inhibitors are necessary. Saliva excretion is greatly influenced by anesthetics. Wagner et al. (1991) proposed a simple method to study saliva secretion in conscious rats and to evaluate sialagogues and sialagogue antagonists.

PROCEDURE

Fed, male Sprague-Dawley rats (200–300 g) are weighed and distributed randomly into groups of 6 animals. Conscious rats are injected i.v., via the lateral tail vein, with either the vehicle or the sialagogue, e. g., substance P (0.3–3 µg/kg in 1 ml saline/kg). The rat's oral cavity is swabbed immediately after i.v. injection by placing and holding a pre-weighed, absorbent foam cube (e. g., 5/16", Texwipe Company, Upper Saddle River, NJ) sublingually for 10 s using a Triceps foam pencil (Texwipe Company, Upper Saddle River, NJ). Conscious rats are restrained during the 10 s collection period by gently holding the animal and opening the mouth using a plastic coated snare, which is looped around the maxillary incisors and drawn back over the animal's head and the hand holding the rat, drawn around in front of the rat and looped around the mandibular incisors. Gentle pressure on the snare opens the rat's mouth allowing the placement of the absorbent cube. Foam cubes are re-weighed immediately

after use. The difference between the initial weight of the cube and the weight of the cube after use represents saliva secreted.

EVALUATION

Data are analyzed with Dunnett's *t*-test that compares several treated groups with a control group. Regression analysis is used to determine dose response and relative potency.

MODIFICATIONS OF THE METHOD

Martinez et al. (1978, 1981) inserted appropriate plastic cannulae into the main excretory ducts of the two submandibular glands in **rats**.

Giuliani et al. (1988) studied the relative contributions of various neurokinin receptors (NK-1, NK-2, NK-3) to the sialogogic response after i.v. application in urethane-anesthetized rats.

Direct cannulation of the glandular duct with polyethylene tubing was performed by Bodner et al. (1983) and Kohn et al. (1992).

Bianciotti et al. (1994, 1996) cannulated the ducts of both the submaxillary and parotid glands in male Wistar rats anesthetized with 10% ethyl urethane. No basal flow of saliva was observed from either gland, however dose-response curves could be established after intravenous injection of sialogogic agents, such as methacholine (0.3 to 10.0 µg/kg), norepinephrine (3 to 60 µg/kg), isoproterenol (1 to 30 µg/kg), methoxamine (30 to 300 µg/kg), substance P (0.3 to 10.0 µg/kg). Atrial natriuretic factor enhanced the salivary response to methacholine, methoxamine and substance P.

Lohinai et al. (1997) determined salivary amylase secretion in conscious rats. Under ether anesthesia a catheter was introduced into the esophagus for salivary juice collection and a cannula was inserted into the jugular vein for infusions. After post-anesthesia recovery, submaximal carbachol infusion was given as a background to obtain steady secretion because of the low basal secretory rate. After application of drugs, volume and amylase were determined in saliva samples collected for 30 min.

Iwabuchi et al. (1994) studied salivary secretion after administration of a muscarinic agonist in **MRL/lpr mice**. Saliva were collected from the floor of the mouth of anesthetized rats with a capillary micropipette every 5 min for 60 min.

A method for the quantitative comparison of atropine substitutes on the salivary secretion of the **cat** has been published by Bülbring and Dawes (1945). Cats anesthetized with pentobarbitone were used. A cannula is tied into Wharton's duct and attached to

a bottle containing tap-water. The tap-water, displaced by the saliva, passes out of the bottle through a tube which actuates a drop timer.

Ekström et al. (1994) used morphometric analyses to study the parotid acinar degranulation in cats after stimulation of the parasympathetic auriculo-temporal nerve.

Izumi and Karita (1994, 1995a, b) investigated the secretory and vasodilator effects of nerve stimulation in the submandibular gland of cats. Cats of either sex were anesthetized with ketamine and a mixture of chloralose and urethane, paralyzed by intravenous injection of pancuronium bromide and artificially ventilated. Blood flow changes in the submaxillary glands and lips of the cats were measured using a laser-Doppler flowmeter. The duct of the submandibular gland was cannulated with a polyethylene cannula inserted distal to the intersection between the chorda lingual nerve and the duct. The amount of saliva secreted in response to nerve stimulation was determined gravimetrically by collecting the saliva in pre-weighed tubes.

Boldyreff (1925) described the preparation of salivary fistulae in the **dog**.

For preparing a **parotid fistula**, a fine sound is introduced through the orifice of the parotid duct, which is found opposite to the largest upper molar tooth, to the depth of 6–8 cm. Around the orifice and at a distance of about 0.5 cm from it, four sutures are laid on the mucosa at equal distances one from the other. After this, a round piece of mucosae, about 1 cm in diameter around the orifice, with the sutures at the edge of this piece, is cut out with small sharp scissors. The duct is then separated from surrounding tissues about 2 cm from the orifice in the direction of its length. Then an opening is made through the cheek into the mouth (from the point half way on the vertical line from the front or the back corner of the eye to the mouth) to the base of the prepared duct. The orifice of the duct is now led outside by pulling out with the forceps. Four sutures on the piece of mucosa are made around it. The piece of mucosa is sutured carefully to the skin with knot sutures. The wound inside the mouth is closed with a continuous suture. The piece of mucosa must be covered daily with vaseline to prevent drying. Sutures must be taken out slowly, beginning 3 days after operation. For the first 10 days after operation it is necessary to produce on the dog an intensive salivary secretion, twice a day, by introducing into the mouth of the animal, dry bread or meat powder or 0.5% solution of hydrochloric acid. Saliva is collected into graduated test tubes.

In a similar way, one can produce a **fistula of the submaxillary or sublingual glands**, usually a common fistula for both glands, because their ducts have a common orifice.

Ogawa et al. (2003) developed a model of chronic parotitis in rats by a direct injection of complete Freund's adjuvant into the unilateral parotid gland via the parotid duct without skin incision.

Lambert et al. (1994) **cultured acinar cells** from lacrimal and submandibular glands as well as epithelial cells from rat small intestine in supplemented, serum-free media and measured the secretory components after treatment with various agents by radioimmunoassay.

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J.2 Esophagus

J.2.0.1

Tunica Muscularis Mucosae of Esophagus In Vitro

PURPOSE AND RATIONALE

The tunica muscularis mucosae preparation of the rat esophagus (Bieger and Triggler 1985; Ohia et al. 1992) has been recommended for evaluation of 5-HT₄ receptor ligands since it possesses a homogeneous population of 5-HT₄ receptors which mediates a well defined relaxant response to 5-HT (Baxter et al. 1991, 1992; Reeves et al. 1991; Waikar et al. 1992; Eglén et al. 1993, 1995; Yang et al. 1993; Gale et al. 1994; Monge et al. 1994; Sagrada et al. 1994; Elz and Keller 1995; Hegde et al. 1995; Cohen et al. 1996, 1998; Wong and Eglén 1996; Nagakura et al. 1999; Takeda et al. 1999).

PROCEDURE

Male Sprague Dawley rats (200–300 g) are sacrificed by asphyxiation with CO₂, and 2 cm segments of intrathoracic esophagus, proximal to the diaphragm, are excised and placed in Tyrode solution of the following composition (mM): NaCl 136, KCl 2.7, MgCl₂·6H₂O 1.0, NaH₂PO₄ 0.4, glucose 5.6, NaHCO₃ 11.9, CaCl₂ 1.8, pH 7.4. The external muscularis propria, containing the outer longitudinal and circular muscle layers of the esophagus, is carefully removed in or-

der to isolate the inner smooth muscle tube of the tunica muscularis mucosae. The tunica muscularis mucosae is suspended in a 10 ml tissue bath containing Tyrode solution at 37°C and aerated continuously with 95% O₂/5% CO₂. Tissues are placed under 2.5 mN tension and are left to equilibrate with Tyrode solution for 60 min (washing every 15 min) prior to starting the experiment. Responses are recorded isometrically using a Hugo Sachs Electronic (Biegestab K30) transducer coupled to a Graphtec (Linearcorder WR3310) 4-channel chart recorder.

Preparation of tunica muscularis mucosae are contracted with carbachol (3 μM, approximate EC₅₀). Concentration-effect curves (relaxation) to 5-HT (or other agonists) are constructed in a cumulative fashion, followed by washout, with a 60-min interval between the first and second curve.

In antagonist studies, the antagonist is incubated with the tissue for 60 min following washout and the second concentration-effect curve is constructed in the presence of the antagonist. Responses are measured as decreases in isometric tension and are expressed as percentage relaxation of the carbachol-induced tone.

EVALUATION

Concentration-Effect Curves and Agonist Potencies

All agonist concentration-effect curves are fitted using a nonlinear, iterative curve fitting program according to the following relationship (Parker and Waud 1971):

$$E = E_{\max}[A]^n / ([A]^n + EC_{50}^n) .$$

This relationship describes curves with a maximal response E_{\max} , half-maximal response EC_{50} (both in terms of molar concentration), and a slope factor determined by the power n . $[A]$ represents agonist concentration and E is response.

Antagonist Potencies

pA₂ estimates of test compounds vs. 5-HT in the rat tunica muscularis mucosae are determined by the method of Arunlakshana and Schild (1959) and computed using Statview II (Brain Power Inc., Calabassas, CA). Concentration ratios are determined using the iterated EC₅₀ values in the absence and the presence of the test compounds.

All remaining pA₂ estimates are determined by the method of Furchgott (1972) using a single concentration of agonist. The method assumes a competitive interaction and is calculated as follows:

$$pA_2 = -\log([\text{antagonist}]/[\text{concentration ratio} - 1])$$

Statistics

CL (95%) and statistical significance of the difference between samples (single comparisons; unpaired Student's *t*-test) are determined using Statview II.

MODIFICATIONS OF THE METHOD

De Boer et al. (1993) divided the **rat** esophagus into two parts, cervical and thoracic, each of a length of 10–15 mm. Both parts were cut longitudinally and pinned on a silicon mat with the outer, striated muscle coat up. After dissection of the striated muscle, the remaining muscularis mucosae was divided into 4 (5 × 2 mm, thoracic part) and 6 (5 × 1.5 mm, cervical part) strips. Strips from different parts showed no differences in pharmacological behavior.

Cohen et al. (1994) found 5-HT₄ receptors in rat but not guinea pig, rabbit or dog esophageal smooth muscle.

Several authors (de Boer et al. 1993, 1995; Kelly and Houston 1996; Lezama et al. 1996; Oriowo 1997, 1998) showed that β_3 -adrenoceptors mediate the relaxation of the rat esophageal muscularis mucosae.

Eglen et al. (1996) studied the functional interactions between muscarinic M₂ receptors and 5-hydroxytryptamine 5-HT₄ receptors and β_3 -adrenoceptors in the isolated esophageal muscularis mucosae of the rat.

Goldhill et al. (1997) investigated the 5-HT₄ receptor modulation of tachykinergic excitation of rat esophageal tunica muscularis mucosae.

The tunica muscularis mucosae of **guinea pigs** was used by various authors:

Yoshida et al. (1993) studied the effect of a gastroprokinetic agent on electrically-evoked contractions in tunica muscularis from isolated guinea pig esophagus.

Watson et al. (1995) investigated the interactions between muscarinic M₂-receptors and β -adrenoceptors in guinea-pig esophageal muscularis mucosae.

Uchida et al. (1998a, b) examined the effect of Ba²⁺ on acetylcholine- and KCl-induced contractions and characterized the endothelin-induced contraction of the guinea-pig esophageal muscularis mucosae.

Malmberg et al. (1991) studied muscle activity of isolated muscle strips from the middle pharyngeal constrictor, the inferior pharyngeal constrictor, the cricopharyngeal muscle, and the cervical esophagus from **rabbits** in organ baths in response to drugs and electrical field stimulation.

Kohjitani et al. (1993, 1996) divided the lower esophagus of rabbits into 3 regions (lower esophagus, transitional zone, lower esophageal sphincter) and studied the influence of anesthetics and peptides

on contractions induced by acetylcholine or electrical field stimulation.

Percy et al. (1997) studied the pharmacological characteristics of rabbit esophageal muscularis mucosae *in vitro*.

Based on studies by Bitar and Mahklouf (1982), Biancani et al. (1987), isolated smooth muscle cells of the lower esophageal sphincter from **cats** were used by Hillemeier et al. (1996) to investigate the influence of protein kinase C on spontaneous muscle tone. Esophagus and stomach from sacrificed cats were removed and opened along the lesser curvature. The location of the squamocolumnar junction was identified, the mucosa was peeled and removed by sharp dissection under a microscope. The underlying circular muscle layer was cut into slices 0.5 mm thick with a tissue slicer. The last slices containing the myenteric plexus, longitudinal muscle and serosa were discarded. The slices of circular muscle were placed flat on a wax surface, and tissue squares were made by cutting twice with a 2-mm blade block, the second cut at right angle of the first. Isolated smooth muscle cells were obtained by enzymatic digestion with collagenase. Agonist-induced contraction of isolated muscle cells was achieved by exposing them to IP₃ and a protein kinase C agonist. The cells were fixed in acrolein at a final concentration of 1%. The length of 30 consecutive intact cells encountered at random was measured with a phase-contrast microscope and a closed-circuit video camera.

Muscle strips from **cat** lower esophageal sphincter were used by Dobрева et al. (1994), Kortežova et al. (1994), Preiksaitis and Laurier (1998).

Uy Dong Sohn et al. (1995) investigated muscle-type-specific signal transduction pathways in esophageal and lower esophageal sphincter circular smooth muscle of cats.

Tokuhara et al. (1993) studied the influence of adrenoceptor agonists on the striated muscle portion of the esophagus by use of isolated strips from **dogs**.

Saha et al. (1993) examined the effects of nitric oxide-containing compounds on **opossum** esophageal longitudinal smooth muscle *in vitro*.

La Rocca et al. (1992) examined the effects of metoclopramide in transverse muscular strips from **pigeon** esophagus.

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PROCEDURE

Adult cats of either sex are fasted overnight. Anesthesia is induced with 15 mg/kg ketamine i.m. and maintained with i.v. infusion of 15 mg/kg/h. The cats can tolerate intubation while continuing to swallow spontaneously or with pharyngeal stimulation. Esophageal motility is continuously monitored by a multi-lumen catheter passed orally. The catheter has a sleeve (Dent 1976) which is positioned within the lower esophageal sphincter, with recording ports 2, 4, 6, and 8 cm above the lower esophageal sphincter and one port just below the sleeve. The recording catheter channels are continuously perfused with distilled water at 0.3 ml/min by means of a pressurized infusion pump. At the recording ports within the esophageal body, the system is able to record a pressure rise of 300 mm Hg/s. Pressures are recorded using transducers. Respiration is continuously monitored with a belt pneumograph placed around the animal's chest. All pressures are recorded on a Beckman eight-channel direct writing chart recorder with input couplers, preamplifiers and amplifiers while simultaneously taped on an eight channel FM tape recorder. Drugs are administered intravenously.

For each esophageal contraction, amplitude is measured at each esophageal level, using mean intra-esophageal pressure as baseline. Onset of contraction is determined as the point of rapid upstroke at each level, and progression of the wave along the esophagus is expressed as the lag of time or delay (in seconds) between two adjacent recording sites. Basal lower esophageal sphincter pressure is measured using intragastric pressure as reference. Maximum lower esophageal sphincter relaxation and lower esophageal sphincter after-contraction are also assessed.

EVALUATION

Statistical analysis is performed by using one-way analysis of variance and a Student's *t*-test where appropriate.

MODIFICATIONS OF THE METHOD

Greenwood et al. (1992) used a similar multi-lumen catheter assembly system in **cats** and registered additionally the electromyogram of the mylohyoid muscle.

Further studies in cats were performed by Preiksaitis et al. (1994) and Lichtenstein et al. (1994).

Using a miniature perfused sleeve/sidehole catheter, Kawahara et al. (1994) measured gastric, lower esophageal sphincter and esophageal pressure in urethane anesthetized **rats**.

J.2.0.2

Esophageal Sphincter In Vivo

PURPOSE AND RATIONALE

Salapatek et al. (1992), Xue et al. (1996) studied the control of esophageal peristalsis and function of the lower esophageal sphincter in anesthetized cats.

Rouzade et al. (1996) monitored manometrically esophageal, lower esophageal sphincter and fundus pressure in conscious **dogs**.

Blackshaw et al. (1995), Blackshaw and Dent (1997) measured responses of the lower esophageal sphincter in urethane-anesthetized **ferrets**.

Smid et al. (1998) studied lower esophageal sphincter function in a model of oesophagitis in ferrets. Oesophagitis was induced by acid (0.15 M HCl) and 1% pepsin infusions in anesthetized ferrets. Lower esophageal sphincter strip responses were measured *in vitro* after various agents and after electrical field stimulation.

Knudsen et al. (1994) measured esophageal pressure with a four-channel, perfused catheter assembly in lightly anesthetized **opossums**.

Further studies in opossums were performed by Harrington et al. (1991), de Arruda-Henry and Uchida-Athanasio (1994).

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J.2.0.3

Permanent Fistula of the Esophagus in the Dog

PURPOSE AND RATIONALE

Esophagostomy was made for the first time by Pavlov (1902) for the purpose of obtaining pure gastric juice from a dog. Pure gastric juice can be obtained in great quantities only after sham feeding from a gastric fistula, so it is necessary that the dog shall have two fistulae: esophageal and gastric fistula. The technique to prepare a permanent gastric fistula in the dog was described by Boldyreff (1925).

PROCEDURE

It is necessary to obtain beforehand a well nourished dog with a gastric fistula. The operation must be done aseptically with the usual anesthesia. The incision on the neck is made on a median line 2–3 cm below the larynx and is 12–15 cm long. The esophagus is found under the trachea and a little to the left. It is separated as little as possible from the surrounding tissue with a knife handle and pulled outside. The esophagus is now divided, cross-section, and the ends of the dissected esophagus are carefully sutured into the respective corners of the wound. In suturing the mucosa of the esophagus with the skin of the wound it is necessary to take into the suture the muscle layer of the esophagus also. After the ends of the esophagus are sutured into the corners of the wound, the part of the wound between them is closed. The wound should be covered with thick antiseptic ointment. After the operation it is necessary to examine the wound daily. The sutures must be taken out gradually beginning five or six days after the operation.

Oesophagotomy can be performed more simply, if the esophagus is not divided but a longitudinal incision, 12–15 cm long is made through its wall. The edges of this incision are sutured together with the edges of the wound in the neck. When the wound is healed this fistula can be temporarily closed during feeding by means of a bandage or with a special de-

vice. The dog can then eat and drink normally and the saliva is not lost through the fistula.

MODIFICATIONS OF THE METHODS

Boldyreff (1925) described also the preparation of crop fistulas in the **rooster**. A longitudinal incision 2–3 cm long is made through the skin in the middle part of the crop. Then, with the aid of a pair of surgical forceps the front wall of the crop is lifted up and an opening about 2 cm long is made in it with scissors. After this a fistular tube is introduced into the crop with the aid of a large hook and, if necessary, the opening of the crop is sutured with one suture tightly around the tube.

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J.3

Gastric Function

J.3.1

Acid Secretion

J.3.1.1

Acid Secretion in Perfused Rat Stomach (Gosh and Schild Rat)

PURPOSE AND RATIONALE

Gosh and Schild (1958) introduced a method for the continuous recording of acid gastric secretion in the rat. The acid secretion can be stimulated by histamine, acetylcholine and gastrin. Moreover, the stimulated secretion can be inhibited by anti-ulcer drugs.

PROCEDURE

(MODIFIED AFTER GOSH AND SCHILD 1958)

Male Sprague-Dawley or Wistar rats weighing about 250 g are used. They are withheld from food, but not from water 18 h prior to the experiment. Four animals are used per dose of test drug or standard. Anesthesia is induced by i.p. injection of 5 ml/kg of 25% urethane solution. Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The jugular veins are then exposed and cannulated with polyethylene tubes bevelled at the tip. The abdomen is opened through a midline incision, the pyloro-duodenal junction exposed and a double perfusion cannula (with two lumina) is introduced through a cut in the duodenum up to the cardiac part of the stomach and secured firmly by placing a ligature around

the pylorus, care being taken not to include blood vessels within the ligature. Using a peristaltic pump, the stomach is perfused continuously with 0.9% NaCl solution at 37°C. In the effluent, pH is measured with a pH-meter and continuously recorded. Gastric secretion is stimulated by continuous intravenous infusion of 100 µg/kg/h of pentagastrin or 3 mg/kg/h histamine hydrochloride or 30 µg/kg/h carbachol. As soon as acid secretion has reached a plateau, test substances or standard are injected intravenously.

EVALUATION

The maximal inhibition of acid secretion (AI_{\max}) and the inhibition of acid secretion during 1 h (AI_t) are calculated.

$$AI_{\max} = (\text{pH}_i - \text{pH}_s) \times (\text{pH}_b - \text{pH}_s)^{-1} \times 100$$

$$AI_t = 100 - F_i \times F_s^{-1} \times 100 \text{ whereby,}$$

pH_b = pH value at basal H^+ secretion

pH_s = pH value at stimulated H^+ secretion

pH_i = highest pH value after administration of test compound

F_s = integral of pH curve over 60 min before administration of test compound

F_i = integral of pH curve over 60 min after administration of test compound.

Moreover, using various doses of the test compound and of a standard, dose-response curves can be established and activity ratios with confidence limits can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method of Gosh and Schild being modified by several authors can be used for standardization of secretagogues, like gastrin, and for evaluation of acid secretion inhibiting anti-ulcer drugs.

MODIFICATIONS OF THE METHOD

Burn et al. (1952) described the evaluation of substances which affect gastric secretion using perfusion of the stomach in anesthetized **cats**.

Lawrence and Smith (1974) described the measurement of gastric acid secretion in the **rat** by conductivity. The stomach of an anesthetized rat is continuously perfused with 2 ml/min of an isotonic (0.308 molar) glucose solution at 37°C. The conductance of a solution depends on the total ion concentration and is therefore not specific for hydrogen ions. Since hydrogen ions have an equivalent conductance nearly

5 times greater than any other ion found in gastric juice and since they are secreted in a far greater concentration than other ions, conductivity measurements can be regarded as a relatively specific measure of hydrogen ions. Using Mullard conductivity cells (type E 791/B) and a commercially available meter (Phillips PW 9501) simultaneous measurements in 6 rats were performed.

Gallo-Torres et al. (1979) described in detail a method for the bioassay of antisecretory activity in the conscious rat with acute gastric fistula with additional collection of the biliary and pancreatic secretion by means of a catheter in the common bile duct. The gastric secretions are collected by gravity via a cannula in the most gravity dependent site of the glandular stomach.

Larsson et al. (1983) described studies in the acutely vagotomized rat. Truncal vagotomy is performed under ether anesthesia by cutting the dorsal and ventral branches of nervus vagus just below the diaphragm. The pylorus is then ligated and a polyethylene catheter (PP 200) is inserted into the duodenum, close to the pylorus. Each animal is placed in a modified Bollman cage and is allowed to recover at least 1 h before the experiment. Gastric juice is collected by free drainage in 30 min samples.

Herling and Bickel (1986) showed that gastric acid secretion in stomach-lumen perfused rats can be stimulated *in vivo* on the subreceptor level by IBMX (phosphodiesterase inhibitor) and forskolin (non-receptor activation of the adenylate cyclase). H^+/K^+ -ATPase inhibitors and H_2 -antagonists show, according to their different modes of action, also a different inhibitory profile in this assay.

Hammer et al. (1992) used anesthetized female Sprague-Dawley rats weighing 200–320 g. After insertion of a tracheal cannula, a 3-mm silicon tubing is placed through the mouth and advanced to the stomach. The tubing is tied to the esophagus at the neck. A 4-mm drainage tube is inserted into the stomach through a laparotomy incision and an incision in the duodenum, and ligated in place at the pylorus. Gastric perfusate (0.9% saline at 37°C) is collected on ice every 5 min for titration to pH 7.0.

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J.3.1.2

Isolated Rat Stomach

PURPOSE AND RATIONALE

The isolated whole stomach of the rat was recommended for evaluation of H_2 -receptor antagonists (Bunce and Parsons 1976).

PROCEDURE

Fed immature rats of either sex weighing 38–42 g are anesthetized with sodium pentobarbitone *i.p.* The abdomen is opened and the esophagus ligated close to the stomach. An incision is made in the rumen of the stomach and the contents washed out with warm Krebs-Henseleit solution. A second incision is then made at the pyloric sphincter and polyethylene cannulae are inserted and tied into the stomach via these incisions. The stomach is rapidly dissected out and placed immediately into a 10 ml organ bath containing Krebs-Henseleit solution at 37°C. The lumen of the stomach is perfused at a rate of 1 ml/min with a modified Krebs-Henseleit solution (without Na_2CO_3 and KH_2PO_4) at 37°C. Both solutions are gassed with 95% O_2 /5% CO_2 . The effluent perfusate from the stomach is passed over a micro dual electrode. The changes in pH are converted to a function of hydrogen ion activity by an an-

tilog function generator and continuously recorded on a potentiometric pen recorder. The drugs are added, in a volume not exceeding 0.5 ml, to the complete Krebs-Henseleit solution bathing the serosal surface of the stomach. After setting up the stomach preparation the basal H^+ output is allowed to stabilize, both under control conditions and in presence of the H_2 -antagonist, before the secretory responses to histamine are investigated. The response to a dose of histamine is assessed by measuring the amount of acid secreted at peak response above the preceding basal level.

EVALUATION

The rate of acid secretion is expressed as $[H^+]$ moles $\times 10^{-8}$ per min. The effect of the antagonist is assessed by measuring the potency of the agonist. An estimate of potency is firstly obtained using the agonist alone, and then a second estimate is obtained in the presence of the agonist-antagonist combination. The potency ratio is calculated according to Finney (1964).

MODIFICATIONS OF THE METHOD

A similar method was described by Szelenyi (1981) and Stanovnik et al. (1988) using the isolated stomach of the **mouse**.

Weigert et al. (1995) evaluated the effect of the opiate receptor antagonist naloxone on vagally stimulated secretion of bombesin-like immunoreactivity, somatostatin and gastrin from the isolated rat stomach which was perfused via the celiac artery with Krebs-Ringer buffer. Vagal stimulation was performed with 1 ms, 10 V and 2, 5, 0r 20 Hz, respectively.

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J.3.1.3

Chronic Gastric Fistula in Rats

PURPOSE AND RATIONALE

A permanent gastric fistula using an especially designed Pavlov's type of cannula in the rat has been described by Lane et al. (1957) and Komarow et al. (1960, 1963). Moreover, the preparation of chronically denervated gastric pouches in the rat has been reported by Alphin and Lin (1959).

PROCEDURE

For a **permanent gastric fistula** in rats Komarow et al. (1960, 1963) developed a stainless steel cannula of the type extensively used in Pavlov's laboratories. The cannula is 10–12 mm in length with an inner bore of 3 mm diameter and a bevelled flange at each end. The inner bore at one flange is threaded to permit insertion of a removable screw which acts as a stopper between experiments. The lumen of the cannula permits a snug fit with a No. 8 French catheter.

Male Wistar rats weighing 280–300 g are used. Under ether anesthesia, the left upper quadrant of the abdomen is shaved and the animal placed on its right side. An incision of 5–10 mm in length is made about 5 mm below and parallel to the lower left costal margin between the parasternal and mammary lines and the deep abdominal muscles are divided. The area of the rumenal wall is then drawn up through the incision. This area should be close to the greater curvature but far enough away from the glandular portion of the stomach to avoid sacrificing secretory mucosa. A small incision is made in the exposed portion of the rumen and a purse string suture is loosely placed around the margin of the opening. The unthreaded flange of the cannula is inserted into the rumen and the suture is pulled tight around the shaft of the cannula and tied. The incision is then closed by two layers of interrupted sutures. The deep abdominal muscle is closed first by a suture at each side of the cannula, using a bit of rumen to ensure a tight fit. The incision is closed with skin sutures.

During the postoperative period of 2–3 weeks, the animals are conditioned to the experimental procedure by keeping them in a restraining cage for several hours each day. Food is withdrawn 18 h prior to the experiment. The animal is placed in a restraining cage, the stopper screw removed from the cannula and the stomach gently washed twice with 2–3 ml warm saline, and 6 cm length of rubber catheter is inserted through the lumen of the cannula. Hourly collections of gastric fluid in a graduated centrifuge tube are then started.

The animal model can be used for physiological studies as well as for standardization of secretagogues and for measuring the inhibition of gastric secretion by anti-secretory drugs.

For preparation of **chronic denervated gastric pouches** according to Alphin and Lin (1959) male Wistar or Sprague Dawley rats weighing 250–350 g are used. Under ether anesthesia, a mid-line incision is made along the abdominal wall and the stomach is gently pulled to the outside by means of a stainless steel hook. Two small clamps are then gently applied along the stomach between the smaller and greater curvature, care being taken not to injure the blood vessels supplying the glandular and the non-glandular portions of the stomach.

An incision is made between the clamps from one end to the other end of the greater curvature with as little involvement of the mesenteries as possible. The mucosa and the muscular wall are sewed up separately. A new opening is made midway along the greater curvature of the denervated pouch into which one end of a stainless steel cannula is secured by double purse-string sutures. The stainless steel cannula is 3 cm in length with an inside diameter of 3 mm. The other end of the cannula is led through a small puncture 1 cm on the left of the midline of the abdomen. The gastric end of the cannula is firmly anchored to the body wall by silk sutures. After closing the midline incision of the body wall by silk sutures, the animal receives 150 MU procaine penicillin. The animals receive a pasted diet for some days and are kept constantly in an air-conditioned room with constant temperature during the recovery period of 2–3 weeks.

For experiments, the animals are deprived of food, but not of water prior to the test, placed in restraining cages and the gastric juice is collected in graduated centrifugation tubes. The amount of secretion into the pouch and the acidity of the fluid are measured after secretagogues, e. g. 20 mg/kg histamine s.c., alone and then two hours later after administration of potential antisecretory drugs.

EVALUATION

The effect on volume and HCl secretion at 30 min intervals after administration of the test compound is compared with the control values. The decrease is expressed as percentage of control.

MODIFICATIONS OF THE METHOD

Larsson et al. (1983) described a modified technique for the chronic fistula rat. Under methohexital anesthe-

sia a plastic cannula is implanted in the rumen of rats close to the glandular part of the stomach wall. At the same time, a polyethylene tube (PE 50) for compound administration is inserted into the upper part of duodenum and fixed to the cannula. The duodenal tube is subcutaneously guided to the neck, where the free end is guided through the skin. One week recovery is allowed before experiments. For the experiment, the rats are placed in modified Bollman cages and 6 consecutive 1-h samples of gastric juice are collected from the gastric cannulae. After the first two collection periods, the test compounds are given intraduodenally.

Altar (1980) described design and method of implantation of a chronic gastric cannula in adult rats.

Johnson et al. (1990) described a chronic gastric cannula for feeding ethanol liquid diet to rats.

Tsukamoto et al. (1984) reported an improved method for intragastric infusion in conscious rats with simultaneous implantation of a cannula into the jugular vein for blood sampling and drug administration.

Rossowski et al. (1997) measured the inhibition of gastric secretion by adrenomedullin, amylin, calcitonin-gene related peptide and their fragments in rats equipped with chronic gastric fistulas.

Bickel et al. (2004) measured sham-feeding in rats with chronically implanted gastric fistula after treatment with an anorectic compound.

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J.3.1.4

Chronic Gastric Fistula in Dogs

PURPOSE AND RATIONALE

A simple technique for preparing chronic gastric fistulas in dogs which does not require placement of cannulae or tubes has been described by Foschi et al. (1984).

PROCEDURE

Male mongrel or Beagle dogs weighing 12–18 kg are used. After thiopental and halothane anesthesia, a round incision (2 cm) is made on the left side of the abdomen below the costal arch. The muscular fascial layers are cut across to either the peritoneum or the abdominal cavity. The stomach is grasped on the greater curvature, as high as possible, and is gently pulled through the abdominal wall. A round excision of the serous-muscular layer (0.5 cm diameter) is made, and then the mucosa is cut and sutured to the serous layer with catgut. A purse string of 3–0 silk is placed 1.5 cm around the gastrostomy hole to invert the opening and to form an antireflux flap. The stomach is also sutured to the peritoneum, the fascial layer, and the skin with catgut. After 5 days, a silver cannula has to be inserted once a week to avoid closure of the gastrostomy.

After 10–14 days, the animals are trained to lie in a cage to their left sides, supported by a 30° angle with the caudal part of the body raised to avoid the passage of gastric juice into the duodenum. For secretion studies, a short plastic tube is inserted through the fistula and gastric juice is collected for periods of 15 min. After 30 min, secretagogues (e. g., pentagastrin 6 µg/kg/min) are given as continuous infusion and samples collected every 15 min.

EVALUATION

The samples are titrated with 0.1 N NaOH to pH 7.0 by a pH meter. The results are expressed as volume (ml), pH, acid concentration (µE/ml), and output (mE/h).

CRITICAL ASSESSMENT OF THE METHOD

The technique described by Foschi et al. (1984) has the advantage of simplicity.

MODIFICATIONS OF THE METHODS

Boldyreff (1925) reported improvements and details of the technique described by Pavlov (1902).

Thomas (1941) described an improved cannula for gastric and intestinal fistulas using a removable flange being inserted separately into the stomach or intestine.

Emås (1960) reported on gastric secretory responses to repeated intravenous infusions of histamine and gastrin in non-anaesthetized and anesthetized cats with gastric fistula. A gastric cannula as well as a duodenal cannula were inserted.

Daly et al. (1980) described an apparatus for intragastric titration in the conscious dog. A titration display unit provides a record of the secretory response both as a digital printout and a bar chart display.

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J.3.1.5

Heidenhain Pouch in Dogs

PURPOSE AND RATIONALE

The preparation of a chronic gastric pouch, as described by Heidenhain in 1878, is one of the classic techniques in experimental surgery. This model has much contributed to the understanding of the physiology and pathology of the stomach and to modern techniques of abdominal surgery in man. The surgical technique has been described again in detail by deVito and Harkins (1957). A preparation of chronic denervated pouches in the rat has been described by Alphin and Lin (1959). Both preparations can be used as pharmacological models for testing antisecretory drugs.

PROCEDURE

Short-haired mongrel dogs or Harrier dogs weighing 15–20 kg are fasted 24 h preoperatively. Intravenous pentobarbital sodium, 30 mg/kg, provides satisfactory anesthesia. It can be supplemented, if necessary, during operation. The abdominal part is shaved with electric clippers, then with a razor. The skin is disinfected with Zephiran-70% alcohol. Sterile drapes are applied to cover the whole surgical field. A mid-line linea alba incision from xiphoid to umbilicus provides excellent exposure and ease for closure. As the posterior sheath is divided, the large ventral fat pad present in dogs should be excised completely. A self-retaining retractor is applied and the stomach is palpated for the absence of food. Then the spleen is displaced, wrapped in warm, moist pads and laid on the ventral wall below the incision.

The stomach is pulled into the operative field. The greater curvature is held at multiple points so that the stomach is stretched out and the line of incision for the pouch is selected. The pouch should be made from the corpus of the stomach so that true parietal cell juice can be obtained. A line projected from the incisura angularis perpendicular to the proximal lesser curvature will generally fall across the junction between corpus and antrum. Appropriate division of the gastric branches of the right gastroepiploic artery at the lower end of the proposed line of transection clears the greater curvature for 1–2 cm. The gastroepiploic artery itself should be sectioned at this site and a long rent formed on the adjacent omentum, else the omentum vessels tend to tear during subsequent manipulations.

An index finger is then inserted through this defect dorsal to the stomach to emerge higher on the greater curvature through the gastrosplenic ligament at the upper end of the proposed line of transection. This portion of the greater curve is cleared for 1–2 cm. Von Petz clamps with their staplings are used to control bleeding and to avoid leakage of gastric content. The stomach should be kept stretched and flattened while the clamps are applied. After division between the staples, any bleeding is controlled and the cut edges of the main stomach and pouch are then oversewn with continuous sutures of black silk. The suture should be of an inverting type. Surprisingly, leakage or excessive adhesions are not a problem when serosal apposition is neglected.

The pouch so formed is about 30% of the corpus volume and provides adequate secretory volume for further studies. A cannula, made of stainless steel, 3 cm long with a bevelled flange threaded at the other

end is placed in the most ventral portion of the pouch through a small incision in the anterior wall. A single purse-string of silk holds it in place. A double sheet of omentum is then wrapped about the pouch and the cannula before being pulled through the abdominal wall, about 3 cm to the left of the mid-line subcostally. It is important that the cannula be held snugly by fascia, otherwise it will readily pull out of the pouch and abdominal wall. The linea alba is closed with a continuous suture of silk and the skin with subcuticular stitches of chromic catgut. On the outside of the cannula a stainless steel jacket is screwed. For further collections, a bag is connected to the cannula by a threaded adapter, facilitating removal for daily drainage of secretions.

At the end of surgery, 300 MU penicillin and 0.5 g of streptomycin are instilled intraperitoneally. Before recovery from anesthesia the dog receives 500 ml 5% glucose in saline intravenously. The same volume is given for 3 days postoperatively together with oral fluid ad libitum. From the 4th day onward, normal food is given. Twenty-four hours secretions are collected every morning and analyzed for free HCl. A period of 7–10 days is required for full recovery from the operation. Special care has to be taken for each animal being kept separately in a suitable cage with mesh bottom.

For pharmacological studies, food is withdrawn 18 h prior to the experiment with water ad libitum. The animals are placed in Pawlow stands and a continuous i.v. infusion of either pentagastrin at a dose of 8 µg/kg/h or histamine at a dose of 0.1 mg/kg/h is administered. Secreted fluid is collected at 15 or 30 min intervals and analyzed for free HCl. As soon as the secretion has reached a plateau, test compounds are given orally or intravenously.

EVALUATION

The effect on volume and HCl secretion at 15 or 30 min intervals after administration of the test compound is compared with the control values. Maximal inhibition is calculated as percentage change against the pre-drug value. Secretin inhibits gastric secretion volume in a dose-dependent manner. Activity ratios for unknown preparations of secretin can be calculated by 2+2 point assays in comparison with the international standard. Gastric secretion can be stimulated by slow continuous infusion of pentagastrin, e. g., (Gastrodiagnost) 8 µg/kg/h.

The Heidenhain pouch technique can also be used for evaluation and standardization of **gastrin analogues**. Two or more doses of the test compound and the standard are given intravenously. Dose-response

curves are established using gastric juice secretion, acid secretion, or pepsin secretion, within two 15-min collection periods as parameters. Activity ratios with confidence limits can be calculated.

For evaluation of **H₂-antagonists**, a continuous infusion of histamine is given over a period of 45 min, starting with 3×10^{-8} M/kg/h. Then, the dose of histamine is doubled and infused for the next 45 min. This procedure is repeated until secretion has reached a plateau. Secretory response curves are constructed in the presence or the absence of a H₂-antagonist at a concentration between 2×10^{-6} and 5×10^{-7} M/kg/h.

To evaluate the **type of gastric acid secretion inhibition**, e. g., cholinergic, histaminergic, or gastrinergic mechanisms, either carbachol (8 µg/kg/h), or histamine (80 µg/kg/h), or pentagastrin (8 µg/kg/h), are administered as continuous i.v. infusion until a plateau of acid secretion is reached. The experiments are performed at weekly intervals with and without the administration of various doses of test compounds. Regression lines of antisecretory effects of various doses of test drugs are constructed and used for calculation of *ID*₅₀ values. Relative potencies versus a standard, e. g., cimetidine, can be calculated by four- or six-point assays.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described a simplified method for isolation of a portion of the stomach as compared to the original method of Heidenhain (1878).

Gastric motility can be measured by balloon manometry of the Heidenhain pouch in the conscious dog. The animals are deprived of food for 18 h before the experiment, but water is allowed ad libitum. A latex balloon, connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB), is introduced through the fistula cannula into the accessory stomach. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of gastric motor activity. Secretin inhibits gastric motility dose-dependent. After injection of gastrin or gastrin analogues, a dose-dependent increase of pressure is noted over a wide dose-range.

Jacobson et al. (1966, 1967) studied gastric secretion in relation to mucosal blood flow by an antipyrine clearance technique in conscious dogs with vagally denervated gastric fundic (Heidenhain) pouches. A vagally denervated fundic pouch was so constructed that the entire arterial blood supply was delivered by the splenic artery. A non-cannulating transducer (electromagnetic flowmeter) and a hydraulic occluder were implanted on the vessel.

The Heidenhain pouch preparation was used by Carter and Grossman (1978), Kauffman et al. (1980) to study the effect of luminal pH on acid secretion evoked by topical and parenteral stimulants and the effect of topical and intravenous 16,16-dimethyl prostaglandin E₂ on gastric bicarbonate secretion.

Baker (1979) and Roszkowski et al. (1986) developed a modified Heidenhain dog pouch preparation for collecting gastric juice exclusively from the pouch during experimental periods but allowed the pouch to be an integral part of the gastrointestinal tract during non-experimental periods. The pouch is prepared using conventional techniques but, instead of being fitted with a simple cannula through the abdominal wall, a three-way cannula is used which provides passage between the exterior orifice, the pouch and the main body of the stomach. By inserting an appropriate adapter, passage is available only to the pouch and not to the main stomach or vice versa.

The Heidenhain pouch technique in dogs has been used for preclinical evaluation of various drugs, such as:

- a histamine H₂ antagonist by Uchida et al. (1993),
- dual histamine H₂ and gastrin receptor antagonists by Kawanishi et al. (1997),
- a 5-HT₄ receptor antagonist by Bingham et al. (1995),
- an other 5-HT₄ receptor antagonist by Wardle et al. (1996),
- inhibition of motilin-induced phase III contractions by pentagastrin by Yamamoto et al. (1994),
- peptide YY by Zai et al. (1996),
- reversible K⁺-competitive inhibitors of the gastric H⁺/K⁺-ATPase by Parsons et al. (1995),
- the antiulcer agent SWR-215 by Kataoka et al. (1997),
- a selective gastrin/CCK-B receptor antagonist by Yuki et al. (1997).

Descroix-Vagne et al. (1993) used Heidenhain pouch preparations in **cats** and **rabbits** to study the effect of perfusion at pH 5.5 on acid and pepsin secretion.

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J.3.1.6 Gastrin Activity

PURPOSE AND RATIONALE

Biological activity of gastrin and its analogues can be determined with a bioassay using the acid secretion as determined by pH-metry (Gosh and Schild 1955, 1958; Barrett 1966; Smith et al. 1970) or by measurement of conductivity according to Lawrence and Smith (1974).

Radioimmunoassays for gastrin have been developed (Jaffe and Walsh 1979) and are available as commercial kits.

PROCEDURE

Male Sprague-Dawley or Wistar rats weighing about 250 g are used. They are withheld from food, but not from water, 18 h prior to the experiment. Four animals are used per dose of test drug or standard. Anesthesia is induced by i.p. injection of 5 ml/kg of 25% urethane solution. Body temperature is artificially stabilized by means of a rectal thermometer and a heated pad. The trachea is exposed and cannulated. The jugular veins are then exposed and cannulated with polyethylene tubes bevelled at the tip. The abdomen is opened through a midline incision, the pyloro-duodenal junction exposed and a catheter with two lumina is introduced through a cut in the duodenum up to the cardiac part of the stomach and secured firmly by tying a ligature around the pylorus. Care must be taken not to include blood vessels within the ligature.

Using a peristaltic pump, the stomach is perfused continuously with a phosphate-citrate buffer at 37°C. In the effluent pH is measured with a pH-meter and continuously recorded. At the beginning, gastric secretion is stimulated by an intravenous injection of 0.5 μ g/kg pentagastrin. Then, the injections are repeated in 1 h intervals with alternating doses between

0.2, 0.4, 0.8, and 1.6 µg/kg of standard or test compound. Since in this dose range linearity of the response to gastrin can be assumed a 2+2 point parallel assay is allowed. After each injection, pH or conductivity is measured for 45 min. The area under the curve after each dosage is evaluated by planimetry.

EVALUATION

Each of 4 animals receives two doses of standard and test compound in the order of a Latin square. The dose differences have to follow a logarithmic scale. The evaluation is performed with the 4 × 4 assay according to Gosh and Schild (1958).

MODIFICATIONS OF THE METHOD

Wan (1977), Chang and Lotti (1986) used CF₁ female mice. The whole stomach was placed in tissue baths and perfused. The effluent of the perfused stomachs was collected at 15 min intervals, and the hydrogen ion concentration was determined by titration with 0.01 M NaOH to pH 7.0, or continuously recording with a pH-meter.

Lotti and Chang (1989) tested the inhibition of gastrin-induced acid secretion by a selective non-peptide gastrin antagonist in mice, rats and guinea pigs.

Black and Kalindjian (2002) reviewed gastrin agonists and antagonists.

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J.3.1.7

Receptor Binding for Gastrin

PURPOSE AND RATIONALE

The gastrin gene in all mammals consists of three exons and encodes a 101 (human, mouse) or 104 (rat) precursor peptide with an N-terminal signal peptide, which is removed at residues 21 or 25 to generate progastrin. The conversion of progastrin to smaller peptides is regulated by multiple mechanisms (Dockray et al. 2001).

Binding to gastrin receptors can be determined in isolated guinea pig gastric glands (Praisman et al. 1983; Gully et al. 1993).

PROCEDURE

The gastric glands of male guinea pigs weighing between 300 and 400 g are isolated according to Berglindh and Obrink (1976). The animals are anesthetized with 30 mg/kg Nembutal. The abdomen is opened and the aorta is cannulated in a retrograde direction. Heparin solution (250 IU/ml) is injected forcefully through the cannula. After one min, the animal is bled through the cannula and a ligature is placed around the mesenteric vessels. The chest is opened and the thoracic aorta clamped. A warm (37°C) phosphate buffered saline solution is pumped into the aorta, whereupon the portal vein is opened to allow a free outflow of the perfusate. By this procedure, most of the solution is forced through the gastric blood vessels. When the stomach appears totally exsanguinated, it is rapidly removed, opened along the lesser curvature and emptied. The cardial and antral regions are discarded. The corpus is rinsed several times with phosphate buffered saline solution and blotted with filter paper.

The thoroughly washed fundic mucosa from two guinea pigs is minced with fine scissors in a standard buffer consisting of 15 mM HEPES, 130 mM NaCl, 12 mM NaHCO₃, 3.0 mM NaH₂PO₄, 2.0 mM MgSO₄, 1.0 mM CaCl₂, 5.0 mM glucose, 4.0 mM L-glutamine, pH 7.4. The minced tissue is washed and then incubated with 0.1% collagenase in the above mentioned standard buffer containing 0.1% bovine serum albumin in a shaker bath at 37°C. After 40 min, the glands are liberated by a series of resuspensions through a 10 ml plastic pipette, filtered through 200 µ nylon mesh (Nytex), washed and collected by centrifugation. The glands are then resuspended in 40 ml of the standard binding buffer containing 0.1% bovine serum albumin at pH 7.4. Two ml aliquots are transferred into 15 ml plastic centrifuge tubes. The amount of protein

contained in fundic glands is determined according to Lowry et al. (1951).

[¹²⁵I]Gastrin binding is measured in the presence of 0.4 ml of gland suspension in triplicate tubes that contain 50 µl of either buffer, unlabeled gastrin (1 µM), or displacers at the desired concentration and 50 µl of [¹²⁵I] gastrin (70 pM final concentration). After 90 min of incubation at 37°C, the mixture is layered over 1 ml ice-cold incubation buffer in microcentrifuge tubes and is centrifuged at 10,000 g for 5 min. The supernatant is discarded and the radioactivity is measured in a γ -scintillation counter.

EVALUATION

IC_{50} values and K_i constants are calculated.

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J.3.1.8

Gastrin Releasing Peptide/Bombesin/Neuromedin

Bombesin is a tetradecapeptide originally isolated from frog skin by the group of Erspamer (Anastasi et al. 1971). Shortly before, Nakashima et al. (1970) isolated from the skin of *Rana pipiens* a peptide called ranatensin because of its contractile effects on smooth muscle. **Gastrin-releasing peptide**, which shares a C-terminus with amphibian bombesin, has been isolated from a variety of mammalian and non-mammalian species (Spindel et al. 1993). **Neuromedin B**, a decapeptide originally isolated from porcine spinal cord

(Minamino et al. 1983), also shows sequence homology to bombesin and gastrin-releasing peptide. **Neuromedin N**, a six amino acid neurotensin-like peptide, shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1990).

The peptides which belong to the bombesin family can be classified in three subgroups according to the sequence of their C-terminal tripeptide: bombesin (-His-Leu-MethNH₂), ranatensin and litorin (-His-Phe-MethNH₂), and phyllitorin (-Ser-Phe MethNH₂).

Bombesin and its homologues are known to affect a wide spectrum of biological processes (Tache et al. 1988; Parkman et al. 1994; Thomas et al. 1994; Konturek et al. 1995; Varga et al. 1994, 1995; Glad et al. 1996).

In the **gastrointestinal tract**, bombesin/gastrin-releasing peptide stimulates hormone and peptide release, stimulates gastric, pancreatic, bile and intestinal secretion, prevents gastric injury, causes smooth muscle contraction and induces epithelial growth (Dietrich et al. 1994; Kortezowa et al. 1994; Liu et al. 1995; Takehara et al. 1995; Wada et al. 1995; Weigert et al. 1995; Roberge et al. 1996; Yegen et al. 1996; Won Kyoo Cho 1997; Azay et al. 1998; Cox et al. 1998; Mercer et al. 1998; Milusheva et al. 1998; Nishino et al. 1998; Alvaro-Alonso et al. 1999; Bozkurt et al. 1999; Ladenheimer et al. 1999; Shahbazian et al. 1999).

Bombesin-related peptides inhibit **food intake** (Kirkham et al. 1994; Ladenheimer et al. 1996; Smith et al. 1997; Plamondon et al. 1998; Rushing and Gibbs 1998; Aalto et al. 1999; Edwards and Power 1999; Horstmann et al. 1999; Merali et al. 1999).

Several **central activities** of neuromedin B and gastrin-releasing peptide are reported, e. g. involvement in the hypothalamic-pituitary system (Pinski et al. 1992; Plamondon and Merali 1997; Garrido et al. 1998, 1999). Moody and Merali (2004) reviewed the behavioral implications of bombesin-like peptides and associated receptors within the brain. Roesler et al. (2004) discussed bombesin receptors as novel therapeutic targets in anxiety disorders. **Autocrine actions** of neuromedin B and gastrin-releasing peptide in small and non-small cell lung carcinomas are described (Gaudino et al. 1988; Siegfried et al. 1999).

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J.3.1.9

Bombesin Receptor Binding

PURPOSE AND RATIONALE

Different types receptors have been described for bombesin-like peptides (von Schrenk et al. 1989; Batty et al. 1991; Rouissi et al. 1991; Severi et al. 1991; Fathi et al. 1993). The mammalian bombesin receptor subfamily of G protein coupled receptors consists of the gastrin-releasing peptide receptor (GRP-R), also called BB2, the neuromedin B receptor (NMB-R), also called BB1, and the bombesin receptor subtype 3 (BRS-3), also called bb3 (Fathi et al. 1993; Mantey et al. 1997; Donohue 1999; Jian 1999). A fourth subtype, bombesin receptor subtype 4, has been isolated from a cDNA library from the brain of the frog, *Bombina orientalis* (Katsuno et al. 1999). A ligand with high affinity to all four receptors has been identified (Pradhan et al. 1998).

Moody et al. (1978) studied the binding of the radiolabeled bombesin analogue [¹²⁵I]bombesin to rat brain membranes. Functional GRP-preferring bombesin receptors were identified in human melano-

noma cells by Pansky et al. (1997). Benya et al. (1995) expressed and characterized clones of human bombesin receptors.

PROCEDURE

BALB/3T3 fibroblasts devoid of gastrin-releasing peptide receptor (GRP-R) and neuromedin B receptor (NMB-R) are selected by clonal expansion after assaying for GRP-R or NMB-R by RNase protection and binding studies (Benya et al. 1992). These BALB/3T3 cells are stably transfected using a full length human GRP-R clone (huGRP-R transfected cells) or a full length human NMB-R clone (huNMB-R transfected cells) (Corgay et al. 1991). In both cases the receptor is subcloned into a modified version of the pCD2 plasmid and transfected using calcium phosphate precipitation. Stable transfectants are isolated in the presence of 800 µg/ml aminoglycoside G-418 (GIBCO, Waltham MA), identified by binding studies, and then maintained in DMEM containing 10% fetal bovine serum and 270 µg/ml G-418. Cells are passaged every 3-5 days at confluence by splitting 1:4.

[¹²⁵I-D-Tyr⁰]NMB (2200 Ci/mmol), [¹²⁵I-GRP (2200 Ci/mmol), and [¹²⁵I-Tyr⁴]Bn (2000 Ci/mmol) are prepared using Iodo-Gen and purified using high pressure liquid chromatography. Binding studies are performed by suspending disaggregated cells in binding buffer containing 75 pM levels of either [¹²⁵I-D-Tyr⁰]NMB or [¹²⁵I-Tyr⁴]Bn and 3 × 10⁶ cells/ml for 30 min at 22°C. Nonsaturable binding of either radiolabeled peptide is the amount of radioactivity associated with transfected cells when the incubation mixture contains either 1 µM NMB or 1 µM Bn.

The ability of various bombesin related agonists or antagonists to inhibit the binding of [¹²⁵I-Tyr⁴]Bn to huGRP-R transfected cells and of [¹²⁵I-D-Tyr⁰]NMB to huNMB-R transfected cells is compared.

EVALUATION

Data are expressed as the percentage of saturably bound reactivity in the absence of nonradioactive peptide. For each experiment each value is determined in duplicate, and the results are expressed as the means ± standard errors of at least 3 separate experiments.

MODIFICATIONS OF THE METHOD

Radulovic et al. (1991) studied biological effects and receptor binding affinities of pseudonapeptide bombesin/CRP receptor antagonists.

Fanger et al. (1993) identified a 63-kDa serum protein that binds somatostatin and gastrin-releasing peptide but not bombesin.

Wada et al. (1997), Ohki-Hamazaki et al. (1999), and Yamada et al. (2000a, 2000b) studied mice lacking the gastrin-releasing peptide receptor, the bombesin subtype-3 receptor or the neuromedin B receptor.

Mantey et al. (2004) and Boyle et al. (2005) described selective ligands for the bombesin receptor subtype 3.

Akeson et al. (1997) identified four amino acids in the gastrin-releasing peptide receptor that are required for high affinity agonist binding.

Ryan et al. (1998) studied the intracellular signaling of the human bombesin orphan receptor BRS-3 by various bombesin receptor agonists and antagonists.

Chave et al. (2000) analyzed the expression of bombesin-like peptides and their receptor subtypes in normal and neoplastic colorectal tissue.

Sun et al. (2000) investigated the presence and characteristics of the functional receptors for bombesin/GRP in human prostate adenocarcinoma specimens by radio-receptor assay and the mRNA expression of the three bombesin receptor subtypes by RT-PCR.

Weber et al. (2000) determined the structure of the mouse gastrin-releasing peptide receptor gene and investigated its basal promoter activity.

Radioligands for the GRP receptor are:

- [¹²⁵I][D⁶Tyr⁶]bombesin-6–13-methylester, [¹²⁵I]GRP, [¹²⁵I][Tyr⁴]bombesin,

for the **neuromedin B receptor**:

- [¹²⁵I]BH-NMB, [¹²⁵I][Tyr⁴]bombesin,

for the **bombesin receptor subtype 3**:

- [¹²⁵I][Tyr⁶,βAla¹¹,Phe¹³,Nle¹⁴]bombesin-6–14 (Alexander et al. 2000).

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J.3.1.10

Evaluation of Bombesin Receptor Antagonists as Anti-Cancer Drugs

PURPOSE AND RATIONALE

Bombesin and gastrin-releasing peptide affect the growth and differentiation of lung epithelium in the fetus and the adult. Gastrin-releasing peptide is an autocrine growth factor for various small cell carcinoma cell lines. Bombesin/gastrin-releasing peptide receptors were also identified in other human cancer tissue (Halmos et al. 1995). Therefore, bombesin- and gastrin-releasing peptide-antagonists were synthesized and tested as antitumor agents (Heimbrook et al. 1991; Qin et al. 1994a, 1995; Thomas et al. 1994; Azay et al. 1996; Casanueva et al. 1996; Halmos and Schally 1997; Moody and Jensen 1998).

The procedure of Qin et al. (1994a) on the inhibitory effect of an bombesin receptor antagonist on the growth of human pancreatic cells *in vivo* and *in vitro* is described as example of various similar studies.

PROCEDURE

Cancer Cell Line

Cancer cells of the CFPAC-1 human pancreatic cell line, originally established from a well differentiated ductal pancreatic adenocarcinoma of a 26-year-old white male with cystic fibrosis, are routinely maintained in a monolayer culture in Costar T75 culture flasks with IMDM medium containing 10% FCS, 0.5 g/liter L-glutamine, 25 mM HEPES, 3.7 g/liter NaHCO₃, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B under humidified 5% CO₂ at 37°C. The cells growing exponentially are harvested by an incubation with 0.25% trypsin-EDTA in calcium- and magnesium-free Hank's balanced salt solution for 5 min at 37°C. For tumor cell implantation, a cell suspension is prepared in serum-free IMDM by repeatedly passing the cells through a G-22 needle; then the cells are diluted to a concentration of 5 × 10⁶ cells/ml.

Implantation of Tumors in Nude Mice

Male athymic BALB/c (nu/nu) 6-week-old mice are housed in a laminar airflow cabinet under pathogen-free conditions throughout the experiments. Three nude mice receive s.c. injections in the flanks with 0.2 ml of cell suspension (1 × 10⁶ cells) and serve as tumor donors. After 4 weeks, the implanted tumors grow to a size of about 5 mm in diameter and are removed from the mice. Tumor samples are dissected free of necrotic tissue and blood vessels and are cut into small fragments of about 8 mm³. Under ether anesthesia, two pieces of tumor fragments are implanted s.c. by trocars on both sides of the flanks for each mouse. The mice bearing the implanted tumors are randomly divided into groups with 10 mice in each group.

The nude mice with implanted tumors start to receive injections of the bombesin antagonist or vehicle 7 days after the tumor cell injection. The treatment is continued for 25 days.

Evaluation of Tumor Growth

During the treatment, the size of the implanted tumors is measured by calipers in each mouse at 3–4 days intervals for 25 days to construct the tumor growth curve *in vivo*. Tumor volume is calculated by the formula: Tumor volume = length × width² × 0.5. Tumor volume

doubling time is defined as the time required for the tumors to grow from 50 mm³ to 100 mm³ for the control group and from 35 mm³ to 70 mm³ for the treatment group, respectively. The tumor growth delay time is estimated as the time difference for the treated tumors and the controls to reach a volume of 70 mm³. At the end of the experiment, the animals are sacrificed by an overdose of ether. The tumors are removed from the animals, weighed, and immediately frozen in liquid nitrogen for measurement of DNA and protein content in tumor tissues.

Determination of DNA and Protein in Tumor Tissue

DNA in tumor tissue is determined by the method of Labara and Paigen (1979) which is based on the enhancement of fluorescence reaction upon binding bis-benzimidazole Hoechst 33528 to DNA in cell nuclei in a high ionic strength solution. Tumors collected in each group are pooled and homogenized in 10 times their volumes in a buffer consisting of 0.05 M NaH₂PO₄, 2.0 M NaCl, and 2 mM EDTA (pH 7.4). Hoechst 33528 is dissolved in the same buffer at a concentration of 1 µg/ml and filtered before use. An aliquot of tumor homogenate (0.4 ml) is suspended in 4 ml of Hoechst 33528 solution, followed by incubation in a dark room for 30 min. The reaction is measured by a fluorescence spectrophotometer at excitation and emission wavelengths of 356 and 492 nm, respectively. Calf thymus DNA type I is used as a standard.

Measurement of Cell Growth in Vitro

The effects of bombesin and the bombesin antagonist on the growth of CFPAC-1 human pancreatic cells *in vitro* is evaluated by direct cell counting and [³H]thymidine incorporation assay.

Direct Cell Counting

CFPAC-1 cells collected from 60–70% confluent cultures are used for this study and seeded to 24-well culture plates (1 × 10⁴ cells/well). After the cells are cultured in IMDM containing 10% FCS for 48 h, the medium is replaced by IMDM supplemented with 2.5% FCS and various concentrations of bombesin, bombesin antagonist, or a combination of both. The same volume of medium but without peptides is added to control wells. Following another 24 h of incubation, the culture is terminated by aspiration of the medium from the wells and washing with PBS (0.5 ml/well). The cells are trypsinized by a 10-min incubation with (0.5 ml/well) 0.25% Trypsin-EDTA. The detached cells are dispersed by repeated pipetting us-

ing a G-22 needle and syringe. The number of cells is counted by an automated electronic cell counter (Coulter Counter Model ZF).

[³H]Thymidine Incorporation Assay

Single cell suspension is prepared in IMDM with 10% FCS and seeded to 24-well culture plates (1 × 10⁴ cells/well). After 46 h of culture, the medium is changed to IMDM (0.5 ml/well) containing 2.5% FCS and various concentrations of bombesin, bombesin antagonist, or a combination of both. The same volume of medium but without peptides is added to control wells. After 24 h of culture [methyl-³H]thymidine (radioactivity 25 Ci/mg) is added to each well (1 µCi/well) to pulse the cells. After a 4-h incubation, the medium is removed from the wells, and the cells are fixed by Camoy's solution (1 ml/well; methanol:glacial acetic acid, 3:1, v/v) for 20 min. After washing 3 times with PBS, the cells in each well are dissolved with 0.5 ml of 0.3 N NaOH for 15 min at room temperature. The cell lysate is collected and mixed with 3 ml of Universal scintillation cocktail. The radioactivity is measured for 1 min by a liquid scintillation beta counter.

Receptor Binding Assay

Receptor binding assay is performed using intact CFPAC-1 cells in monolayer cultures. Tyr⁴-bombesin is labeled with ¹²⁵I-Na using a Bio-Rad enzyme-bead iodination kit. Mono-¹²⁵I-Tyr⁴-bombesin is purified by high performance liquid chromatography resulting in a specific activity of ¹²⁵I-Tyr⁴-bombesin of about 2000 Ci/mmol. CFPAC-1 cells are seeded to 24-well culture plates (1 × 10⁴ cells/well) and cultured with IMDM containing 10% FCS for 48 h. The cells in subconfluent culture are washed once with serum-free IMDM supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM MgCl₂, 1 mM EGTA, 10 mM monothio-glycerol, 0.25 mM phenylmethylsulfonyl fluoride, aprotinin 10000 kallikrein inactivator units/liter, and 0.1% bovine serum albumin (pH 7.5), followed by an incubation for 2 h at 22°C with the same medium containing (0.5 nM) ¹²⁵I-Tyr⁴-bombesin in the presence or absence of various concentrations of bombesin, bombesin antagonists or structurally unrelated peptides. The binding reaction is terminated by adding 0.5 ml of ice-cold medium to each well. After washing four times with ice-cold PBS (pH 7.4) the cells in each well are dissolved with 0.5 ml of 0.3 N NaOH. The resultant cellular lysate is collected from each well for measurement of radioactivity by a gamma counter.

EVALUATION

All data are expressed as the mean \pm SEM of duplicate or triplicate observations from at least 2–3 repeated experiments. Mean values between the treatment and control group are analyzed by the Student *t*-test, Mann Whitney *U*-test, or one-way analysis of variance.

Data from receptor binding assays are analyzed by a ligand-PC computerized curve-fitting program created by Munson and Rodbard as modified by McPherson (1985) to determine the types of binding sites, the dissociation constants (K_d) and the maximal binding capacity of receptors (B_{max}).

MODIFICATIONS OF THE METHOD

Similar studies, mostly by Schally's group, as with tissue derived from human pancreatic cancer were performed with nitrosamine-induced pancreatic cancers in hamsters (Szepeshazi et al. 1993, 1994, 1999), with human prostate cancer (Pinski et al. 1993a, b), with rat prostate cancer (Pinski et al. 1994a), with human gastric cancer (Halmos et al. 1994; Qin et al. 1994b), with human small-cell and non-small-cell lung carcinoma (Pinski et al. 1994b; Moody et al. 1996; Koppa et al. 1998; Kiaris et al. 1999a), with human breast cancer (Yano et al. 1994; Miyazaki et al. 1998; Kahan et al. 2000), with mouse mammary cancer (Szepeshazi et al. 1992, 1997), with human colon cancer (Radulovic et al. 1994), with human glioblastoma (Kiaris et al. 1999b), and with human renal adenocarcinoma (Jungwirth et al. 1998).

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J.3.2

Mucus Secretion

J.3.2.1

Isolated Gastric Mucosal Preparation

PURPOSE AND RATIONALE

Main and Pearce (1978) described an isolated gastric mucosal preparation from rats for studying the pharmacology of gastric secretion and the synthesis or release of endogenous substances.

PROCEDURE

Rats of either sex weighing 100–120 g or guinea pigs weighing 400–600 g are anesthetized with 60 mg/kg

pentobarbitone s.c. The abdomen is opened along the midline and the stomach exteriorized. The nonglandular portion is removed and the stomach rinsed with mucosal solution, containing 136 mM NaCl, 5 mM KCl, 3.6 mM CaCl₂·6H₂O, 1.2 mM MgCl₂·6H₂O, and 16.7 mM glucose. The muscular layer overlying the nonantral region is separated from the mucosa by blistering. The tip of a fine needle (27 gauge) is inserted just below the muscle and mucosal solution injected between the layers. The process is repeated as many times as is necessary to blister the whole area. Fine scissors are then used to cut the muscle along the greater curvature and then parallel to the cut edge. The muscle sheet is pulled back to expose the mucosa. The stomach is then removed, opened by cutting along the greater curvature, and rinsed with mucosal solution.

Two pieces are obtained from one stomach and each is placed, mucosal surface inwards, over a 1-cm²-opening on a polyethylene vessel (titration cup). Both preparations, one each from the ventral and dorsal surfaces, are stretched lightly over the cup and tied in place. Each tissue is placed in an organ bath, at 37°C, containing 35 ml of serosal solution (110 mM NaCl, 5 mM KCl, 3.6 mM CaCl₂·6H₂O, 1.2 mM MgCl₂·6H₂O, 26 mM NaHCO₃, and 16.7 mM glucose) being gassed with a 95% O₂/5% CO₂ mixture. The mucosal surface is superfused by means of a peristaltic pump at a rate of 0.5 ml/min with an unbuffered solution of similar ionic composition and gassed with 100% O₂. The volume of solution on the mucosal side is kept constant by suction via the same pump using larger diameter tubing, varying between 1.6 and 2.0 ml. This small volume is used in order to follow changes in acid secretion more closely. Secretion is recorded continuously via a dual microelectrode in the mucosal solution connected with a potentiometric pen recorder. The H⁺-ion concentration is noted every 15 min and expressed as apparent secretion rate. Drugs are added to the serosal solution in volumes not exceeding 1 ml. Contact times for drugs and hormones (e. g., gastrin or histamine) are between 30 and 60 min. Recovery periods before the next response are between 45 and 60 min. Responses are readily reversible on replacing the solution in the bath with fresh, warmed serosal solution.

EVALUATION

The secretory response is calculated as the increase in secretion rate at the peak of the response over the preceding basal value. Dose-response curves and time-response curves are established for histamine, gastrin,

and methacholine. The effects of drugs which inhibit acid secretion are evaluated by adding them either at the peak of a response, or 15 to 60 min prior to the secretagogue.

MODIFICATIONS OF THE METHOD

Wan et al. (1974) used the fundic glandular portion of the rat stomach mounted onto a glass tube without removing the muscular layer to study the inhibition of *in vitro* stimulated gastric acid secretion by a histamine H₂-receptor antagonist.

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J.3.2.2

Primary Culture of Rat Gastric Epithelial Cells

PURPOSE AND RATIONALE

Zheng et al. (1994) described an *in vitro* model for evaluation of antisecretory agents using primary cultures of rat gastric epithelial cells.

PROCEDURE

Gastric mucosal cells from 1- to 2-week-old Sprague-Dawley rats are isolated according to Terano (1982). Gastric mucosal surface is washed thoroughly with sterile cotton and Hank's balanced salt solution (HBSS) and then rinsed with HBSS before being minced into approximately 1-mm³ pieces. The minced tissues are incubated in HBSS containing 0.1% collagenase and 0.05% hyaluronidase at 37°C in a shaking water bath for 60 min, then pipetted several times and filtered through a sterile nylon mesh. The filtrate is washed twice with HBSS by centrifugation (200 g for 5 min) and resuspended in Coon's modified Ham's F-12 culture medium containing 100 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, 15 mM HEPES, 2 µg/ml fibronectin, and 10% fetal bovine serum. Cells are seeded at a density of 1.5–2 × 10⁵ or 1–2 × 10⁴ cells/cm² directly onto either 96-well plates or 6-well plates, and maintained in a Steri-Cult incubator at 37°C in a humidified atmosphere with 5% CO₂. The medium is changed daily. The confluent monolayers are formed after 4–5 days in 96-well plates.

Drug Treatment

To measure the effects of individual drugs on the viability of gastric mucosal cells, cells are incubated with 0.02–5 mg/ml drugs in culture medium for either 2 or 48 h for the uptake of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) or up to 8 days for the colony-forming efficiency assay. To measure the cytotoxicity of drugs, e.g., indomethacin, and acidified medium to gastric mucosal cells, cells are incubated in either serum-free medium containing 0.5–10 mM indomethacin for 1 h, or pH 3.5 at for 10–30 min.

To study the effect of various antacids, e.g., aluminium hydroxide or sucralfate preparations, cells are incubated for 1 h in culture medium containing these drugs. The drug suspensions are then aspirated away, followed by another hour of treatment with 3.5 mM indomethacin. Alternatively, cells are treated with drugs and 3.5 mM indomethacin concurrently for 1 h. The effects of antacids on acid-induced damage are investigated by incubating the cells with the agents for 2 h and then exposing them to pH 3.5 medium for 10 to 30 min.

MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-Tetrazolium Bromide) Colorimetric Assay

Cells in 96-well plates are treated with the different drugs and then incubated in 100 µl of culture medium containing 10 µl of an MTT stock solution (5 mg/ml) for 4 h at 37°C according to Mosmann (1983). Following the incubation, 100 µl acid-isopropanol (0.04 N HCl in isopropanol) is added to the wells and incubated overnight at room temperature. The color changes are recorded at 540 nm on a microplate reader. To exclude the disturbance of precipitates in some samples, the samples are centrifuged and only the supernatants are read. For each experiment, a standard curve is generated by measuring the relationship of absorbance to a series of viable cell numbers.

Neutral Red Uptake Assay

Neutral red is prepared as a 1% stock solution in distilled water and diluted to 0.035% in HBSS immediately before each experiment. The cells are treated with the drugs and then stained with 0.1 ml of 0.035% neutral red for 30 min (Parish and Müllbacher 1983). The stain is discarded and the cells are washed twice in HBSS before the addition of 200 µl/well of acidified alcohol solution (50%, v/v, ethanol/water, containing 0.5% acetic acid). After a 2 h incubation period at room temperature, the color changes are measured in a microplate reader.

Colony-Forming Efficiency Assay

The cells are seeded into 6-well plates and incubated in culture medium containing 0.02 to 5 mg/ml of individual drugs for 8 days. Cells are then fixed in 10% formalin and stained with 1% aqueous crystal violet (Sundqvist et al. 1989). Colonies formed on each well are counted (crystal violet stains cell nuclei) and compared with those formed on drug-free wells.

EVALUATION

Data are expressed as mean \pm standard error. A one-way analysis of variance followed by Scheffe's post hoc test is used to test the significance between control and drug-treated samples. Differences are considered significant at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Buchan et al. (1993) used cultured human antral epithelial cells enriched for D cells to study the effect of cholecystokinin and secretin on somatostatin release.

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J.3.3**Gastric Motility****J.3.3.1****Measurement of Intra-gastric Pressure in Rats****PURPOSE AND RATIONALE**

Gastric motor activity can be measured by recording intra-gastric pressure in anesthetized rats (Holzer 1992).

PROCEDURE

Sprague-Dawley rats of either sex weighing 220–240 g are deprived of food for 20 h prior experimentation but are allowed free access to tap water. After induction of anesthesia by i.p. injection of phenobarbital sodium (0.92 mmol/kg), the trachea is cannulated

to facilitate spontaneous breathing. The left carotid artery is cannulated and connected to a pressure transducer to monitor mean arterial blood pressure. The left jugular vein is cannulated for i.v. injection of drugs and for continuous infusion of physiological saline at a rate of 0.6 ml/h to avoid dehydration of the animals. Intra-gastric pressure is measured by a catheter (outer diameter 1.9 mm) passed down to the stomach via the esophagus. The position of the catheter tip in the corpus region is verified at the end of each experiment. The catheter has two side holes in its tip segment and is continuously perfused with physiological saline at a rate of 0.6 ml/h. To record intra-gastric pressure, the catheter is connected to a pressure transducer.

EVALUATION

The gastric motor effect is quantitated by calculation of the area under the curve versus baseline intra-gastric pressure, measured immediately before injection of the stimulant (e. g., neurokinin A). To test inhibitors, the area under the curve after the stimulant is set as 100% and the effect of application of the inhibitor with the stimulant is calculated as percentage.

MODIFICATIONS OF THE METHOD

Lotti et al. (1986) described a simple mouse assay for the *in vivo* evaluation of cholecystokinin antagonists which is based on visual determination of the gastric emptying of a charcoal meal.

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J.3.3.2**Isolated Smooth Muscle Preparation of Guinea Pig Stomach****PURPOSE AND RATIONALE**

Boyle et al. (1993) described a novel smooth muscle preparation from the guinea pig stomach for characterization of CCK receptors by use of selective antagonists.

PROCEDURE

Adult male guinea pigs weighing 330–400 g are sacrificed and the stomach rapidly removed. The fundus is discarded and the stomach is opened along the

greater curvature, pinned on a Petri-dish with the mucosa pointing up. The mucosal and submucosal layers are removed by dissection to reveal the underlying smooth muscle layer. Strips of circular muscle (2 × 25 mm) from the corpus region of the stomach are obtained by cutting inwards, following the striations of the lesser curvature. Muscle strips are mounted in siliconized 3 ml organ baths containing Krebs-Henseleit solution. The buffer is modified to include 5 μM indomethacin. The solution is maintained at 37°C and continuously gassed with a mixture of 95% O₂/5% CO₂. Isometric contractile responses are measured with force-displacement transducers (e.g., Grass FT.03) and recorded on a polygraph (e.g., Mark VII Graphtec Linearorder).

Tissues are placed under 1 g tension and allowed to equilibrate for 30 min after which time they are contracted with a submaximal dose of carbamylcholine (10 nM). Using a 12 min dose cycle, concentration-response curves are established for agonists, e.g., gastrin, pentagastrin, CCK and CCK-analogues. For studies with antagonists, the tissues are exposed to antagonists for 15 min before re-exposure to agonists. The addition of 5 μM indomethacin removes the spontaneous activity due to the inherent myogenic tone existing in the tissue, but leaves the responses to the agonists unaffected.

EVALUATION

Contractile responses to exogenously applied agonists are expressed as absolute changes in tension and are transformed as a percentage of the maximal response achieved for that agonist in order to obtain potency values. EC₅₀ values are obtained graphically for individual concentration-response curves. Responses to agonists in the presence of antagonists are expressed as a percentage of the control maximum response obtained in the same tissue preparation. Agonist concentration-response curves in the absence and presence of increasing concentrations of antagonists are obtained. The method of Arunlakshana and Schild (1959) is used to provide Schild plots of the data, and to obtain affinity constants for the antagonists.

MODIFICATIONS OF THE METHOD

Riazi-Farzad et al. (1996) described an improved preparation of the **isolated rat stomach fundus strip** based on the finding that the majority of the contractile response to 5-HT and carbachol was present in the left ventral longitudinal quartile of the tissue.

Van Nueten et al. (1978), Reyntjens et al. (1984), Schuurkes et al. (1985), Kishibayashi and Karasawa

(1998) used an **isolated gastroduodenal preparation of the guinea pig**. After ligation of the esophagus, the stomach was filled with 20 ml saline and suspended in 200 ml of oxygenated Krebs-Henseleit solution, maintained at 37°C. The duodenum was cannulated and connected with an ultrasonic transit time transducer to record changes in intraluminal volume and with a bottle of saline to ensure constant hydrostatic pressure of 6 cm H₂O. Spontaneous phasic activity was always present on the stomach and recorded as rhythmic changes in gastric volume. Gastric peristaltic waves either stopped at the pylorus or were propagated to the duodenum. Antroduodenal coordination was quantified as the relative number of antral waves that were propagated to the duodenum. After 30 min stabilization, drugs were added in varying concentrations and the effects followed for 30 min. Three parameters were determined: (1) contractile amplitude, expressed as milliliters of expelled volume; (2) frequency, measured as number of contractions per minute; (3) percentage of antroduodenal coordination.

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J.3.4

Absorption

J.3.4.1

Measurement of Gastric Absorption of Drugs in Rats

PURPOSE AND RATIONALE

Although the stomach is not the prime absorptive site of drugs, the absorption of some drugs from the gas-

tric mucosa has been established (Doluisio et al. 1969; Welling 1977). Worland et al. (1983) described an *in situ* gastric pouch technique for direct measurement of the gastric absorption of drugs in the rat.

PROCEDURE

Male Wistar rats weighing 220–300 are anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital. A cannula is placed in the jugular vein for administration of heparinized saline (3000 IU/kg) and whole blood replacement during the experiment. The replacement blood is collected from heparinized donor rats immediately prior to the experiment. Another cannula is placed into the carotid artery of the experimental animal for the collection of systemic blood samples. A midline incision is made in the abdominal wall. Double ligatures are placed on the superior epigastric vessels and transverse incisions are made between the ligatures. The branches of the right gastroepiploic veins and arteries are then ligated using surgical silk and the gastrohepatic ligament between the stomach and the posterior surface of the left hepatic lobe and the caudal hepatic lobe is separated. The gastrosplenic mesentery is also severed. A strip of gauze is employed to keep the liver lobes out of the surgical field. Using appropriately sized surgical silk, double ties are placed around the short gastric vessels and the esophagus, which are then severed between the ties. The pylorus and pyloric vessels are then ligated and an incision is made in the forestomach to allow the removal of gastric contents.

The pouch is then rinsed with warm saline until clear and the remaining fluid is removed using an adsorbent tissue. A Luer adapter modified from a three-way tap is tied into the incision to enable drug administration via a syringe. The gastric pouch is then transposed to the right of the animal exposing the left gastric vein. The vein is cannulated above the junction with the lienal (splenic) vein using a 21-gauge needle connected to a 15-cm length of polyethylene tubing (i.d. 0.75 mm, o.d. 1.45 mm). A small Oxford clamp with foam rubber insets is employed to prevent dislocation of the cannula during the changing of the sample vials. The drug is administered into the stomach and blood draining from the gastric pouch is collected over timed intervals. To determine the volume (ml) of blood collected, the venous effluent is weighed and hematocrit (HCT) readings are taken for each sample and converted to units using the formula:

$$\text{Volume (ml)} = \frac{\text{mass blood collected (g)}}{\text{HCT} \times \text{blood cell density} + (1 - \text{HCT}) \times \text{plasma density}} \cdot$$

Rat blood cell and plasma density measurements are obtained from five determinations from four rats, blood samples being separated at 3000 g for 10 min. Samples from the gastric pouch are kept on ice until the plasma can be separated by centrifugation at 3000 g for 10 min and the plasma frozen until assay.

Blood replacement is delivered at a rate of 0.7 ml/min from a gently oscillating reservoir using a peristaltic pump.

The compound to be tested is administered in a volume of 0.5 ml to the gastric pouch. Plasma levels are determined with a method specific for the compound under investigation. Plasma samples are collected at 4 min intervals over a period of 30–60 min. At the end of the experiment, the fluid in the gastric pouch is collected for determination of the dose remaining in the stomach.

EVALUATION

Mean values \pm standard deviation of plasma concentration are calculated from 4–6 experiments and plotted versus time to demonstrate the absorption profile.

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J.3.5

Antacid Activity

J.3.5.1

Evaluation of Antacids

PURPOSE AND RATIONALE

Antacids have been used for the treatment of gastroduodenal ulcerations since a long time (Konturek 1993). The main action of antacids is to reduce the acidity of the gastric content through neutralization and increasing intragastric pH. Since pepsin is not active at higher pH levels, antacids reduce peptic activity (Goldberg et al. 1968) and may also adsorb pepsin (Sepelyak et al. 1984). Binding of bile salts (Clain et al. 1977) by antacids may also have a beneficial influence on peptic ulcer disease. Most antacids contain magnesium and aluminum hydroxide, in some cases also calcium carbonate and sodium bicarbonate. The acid-neutralizing potency can be measured *in vitro*.

PROCEDURE

0.1 M HCl is added to the antacid to be tested. The acid neutralizing capacity is defined as the amount of 0.1 M HCl that can be added to a liquid antacid without reducing the pH of the mixture below pH 3.0. The determination of the acid neutralizing capacity of antacid tablets is performed *in vitro* by stirring a mixture of crushed tablets and water. The time at which all antacid is consumed is much longer for antacid in tablet as compared with the same amounts of antacid in liquid form. Magnesium hydroxide is very insoluble in water, but is readily soluble in hydrochloric acid. In combined preparations, the magnesium hydroxide reacts first to produce an almost immediate neutralizing effect and an increase of the pH within a few minutes. Aluminum oxide, on the other hand, has a weaker antacid activity, reacting more slow with acid (Fordtran et al. 1973; Richardson et al. 1988).

EVALUATION

In vitro titration curves of 0.1 M HCl with 5 ml liquid antacid or one tablet are measured over 3 h and compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

Acid neutralizing potency may be not the only factor which contributes to the therapeutic effect of antacids. Damage and protection in the stomach are essentially represented by acid secreted by the parietal cells and by bicarbonate released by the surface epithelial cells and mucous neck cells. Among various neurohumoral factors most important in the bicarbonate-secretion appear the prostaglandins, mainly of the E series. Mucosal bicarbonate secretion may be stimulated through the activation of prostaglandins by aluminum hydroxide containing antacids. Aluminum containing antacids were found to protect against mucosal damage due to topical irritants and against stress or aspirin induced lesions. Gastroprotection of aluminum-containing antacids has been attributed to the biological activity of nitric oxide interacting with mucosal prostanoids on the mucosal microcirculation. Therefore, these antacids were found to be active in various experimental ulcer models and to exert cytoprotective activity against ethanol induced gastric injury in rats (Szylenyi et al. 1983; Domschke et al. 1986; Hollander et al. 1986; Konturek et al. 1989, 1992; DiJoseph et al. 1989; Vergin and Kori-Lindner 1990). Konturek (1993) reported that aluminum hydroxide containing antacids protect growth factors, involved in the healing of ulcers, against acid degradation.

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J.3.6

Inhibition of HCl Secretion

J.3.6.1

Anticholinergic Activity

J.3.6.1.1

General Considerations

Gastric motility and tonus, as well as gastric secretion are stimulated by cholinergic impulses. Anticholinergic compounds, such as the *Belladonna* alkaloid atropine, were the first drugs used in treatment of gastric ulcers. The doses that are necessary to reduce acid secretion also decrease mucus secretion and cause side effects, such as dry mouth, increase in heart rate and ocular disturbances. Inhibitors of specific muscarinic

receptors responsible for gastric acid secretion were found. Receptors for acetylcholine which play a major role in central and peripheral transmission have been studied extensively (Karlin et al. 1976; Hulme et al. 1990; Jones et al. 1992; Keibadian and Neumeyer 1994).

J.3.6.1.2

Acetylcholine Receptor Binding

PURPOSE AND RATIONALE

The search of specific muscarinic antagonists for inhibition of gastric acid secretion led to the discovery of pirenzepine, a tricyclic compound, originally tested as a psychotropic agent. This compound has a greater gastrointestinal selectivity than other muscarinic antagonists (Carmine and Brogden 1985; Longdong 1986). The activity of pirenzepine has been localized to M_1 receptors.

This, and the involvement of acetylcholine in many physiological processes, has stimulated the research on the various types of muscarinic receptors. At present, 5 types have been described but further subdivisions can be envisaged (Hulme et al. 1990; Jones et al. 1992; Keibadian and Neumeyer 1994; Caulfield and Birdsall 1998; Alexander et al. 2001; Eglen et al. 2001; Ma et al. 2004). The subtypes of muscarinic receptors have been characterized by the use of organs with a predominant subtype receptor population, e. g., rabbit vas deferens stimulated with electric impulses for M_1 , electrically stimulated left atria from guinea pigs for M_2 , and longitudinal smooth muscle preparations of guinea pig ileum or salivary gland of the rat for M_3 (Doods et al. 1987; Lambrecht et al. 1993), or by the use of selective antagonists (Pitschner et al. 1989; Richards 1990; Svensson et al. 1992,) or agonists (Lambrecht et al. 1993). Further characterization has been achieved by studies of the transduction mechanisms (Brown and Brown 1984; Brown et al. 1985; Parekh and Brading 1992) and by voltage clamp techniques (Bernheim et al. 1992). The five muscarinic receptor subtypes are referred to as M_1 – M_5 . The odd-numbered receptors (M_1 , M_3 , M_5) couple efficiently, through $G_{q/11}$, to activation of phospholipase C, which initiates the phosphatidyl-inositol turnover response. The even-numbered muscarinic receptors (M_2 – M_4) inhibit adenylyl cyclase activity via activation of the G_i class of G proteins.

The genes for the muscarinic acetylcholine receptor subtypes have been cloned and expressed in Chinese ovary hamster cells (Buckley et al. 1989; Dörje et al. 1991) or in fibroblasts (Kashihara et al. 1992).

Many organs, e. g., rat brain (Luthin and Wolfe 1984; El-Fakahani et al. 1986) have been used for studies on acetylcholine receptor subtypes. Only the method using Chinese ovary hamster cells is presented here.

PROCEDURE

Preparation of Plasmid DNA

The coding sequences of the m_1 , m_2 , m_3 , m_4 , and m_5 receptors are derived from a human genome library. The cDNAs are inserted into the Okayama/Berg pCD or pCD-PS expression vector (Bonner et al. 1987, 1988; Buckley et al. 1989). Plasmid DNA is isolated by two sequential density gradient centrifugations through CsCl (Maniatis et al. 1982).

Cell Culture

Chinese hamster ovary cells are incubated at 37°C in a humidified atmosphere (5% CO_2) as a monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml each of penicillin G and streptomycin, and 4 mM glutamine.

Transfection Procedures

Cells are transfected using a modified calcium phosphate procedure (Chen and Okayama 1987) involving the use of cotransfected pcDneo as a selectable marker. Selection with the neomycin analog G 418 (600 μ g/ml; Gibco NY) is started 72 h after transfection and continued for 2–3 weeks. Media are changed every 3 days. Clonal cell lines are obtained by single cell cloning and assayed for [3 H]NMS (N-methylscopolamine hydrochloride) binding capacity.

Membrane Preparation

Cells are grown to about 80% confluency, scraped into ice-cold binding buffer and homogenized for 30 s using a Brinkman homogenizer (setting 5). Membranes are pelleted at 16,000 g for 15 min and rehomogenized. Membrane protein is determined using a Bio-Rad protein assay dye reagent. Membranes are stored frozen at –80°C before use.

Radioligand Binding Studies

All membranes, drugs, and radioligands are made up in binding buffer consisting of 25 mM sodium phosphate (pH 7.4) containing 5 mM magnesium chloride. The assays are performed in 1 ml total volume. Final membrane concentrations are: m_1 6 μ g/ml; m_2 10 μ g/ml; m_3 5 μ g/ml; m_4 3 μ g/ml; and m_5 4 μ g/ml. In [3 H]NMS saturation experiments, 8–10 different concentrations

of the radioligand (2–1400 pM) are employed. For displacement experiments, [³H]NMS in a concentration of 150 pM and 10 different concentrations of the displacer are used. Specific binding is defined as the difference in [³H]NMS binding in the absence and presence of 1 μM atropine. Alternatively [³H]pirenzepine is used. Incubations are carried out at 22°C for 3 h. Assays are terminated by filtration through a Brandell cell harvester onto Whatman GF/C filters. Membranes are washed three times with 5 ml of ice-cold binding buffer before being dried. They are transferred to 10 ml of scintillant (New England Nuclear Aquasol) and counted in a LKB β-counter.

EVALUATION

Data from direct binding experiments are fitted to the equation:

$$\alpha = (B_{\max}x^n/k)/(1 + x^n/k)$$

to derive the Hill coefficient n and to:

$$\alpha = (B_{\max}x/K_D)/(1 + x/K_D)$$

to obtain the dissociation constant K_D and the total number of binding sites B_{\max} ($\alpha =$ [³H]NMS specifically bound; $x =$ [³H]NMS concentration).

Data from displacement experiments are fitted to the equation:

$$\%[{}^3\text{H}]\text{NMS bound} = 100 - [100x^n/k/(1 + x^n/k)]$$

to obtain the Hill number n and to:

$$\%[{}^3\text{H}]\text{NMS bound} = 100 - [100 \times IC_{50}/(1 + x/IC_{50})]$$

to derive the IC_{50} value ($x =$ concentration of the cold inhibitor).

K_i values are calculated by the method of Cheng and Prusoff (1973):

$$K_i = IC_{50}/(1 + L/K_D)$$

where L is the concentration of the radioligand, IC_{50} is the concentration causing 50% inhibition of the specific radioligand binding and K_D the dissociation constant of the radioligand receptor complex. Data are analyzed by a non-linear least-squares curve fitting procedure.

Results are expressed as mean values \pm SEM of n experiments. Statistical significance is assessed using Student's t -test or Scheffé's method. $P < 0.05$ is accepted as being significant.

MODIFICATIONS OF THE METHOD

The selectivity towards muscarinic receptor subtypes can be tested by radioligand binding assays using either selective ligands or tissues possessing only one receptor subtype (Giachetti et al. 1986; Pitschner et al. 1989; Bickel et al. 1990). M_1 -receptors from bovine cortex which has also M_2 - and M_3 -receptors are tested with the M_1 -selective radioligand ³H-pirenzepine. M_2 -receptor from porcine heart possessing only this receptor type can be tested with the unselective ligand ³H-N-methylscopolamine. M_3 -receptors from rat submaxillary gland are likewise labelled with the unselective ligand ³H-N-methylscopolamine, because this subtype is present predominantly in this tissue.

Measurement of the contractions of rabbit vas deferens after electrical stimulation was used to study the effects of prejunctional M_1 heteroreceptors and postjunctional M_2 receptors (Eltze et al. 1988; Dörje et al. 1991), of guinea pig atria for M_2 -receptors and of guinea-pig ileum for M_3 -receptors (Lambrecht et al. 1989, 1995). Cardiac muscarinic $M_{2\alpha}$ receptors have been discussed (Wess et al. 1988).

Coexistence of M_2 and M_3 subtypes of muscarinic receptors in canine colonic circular smooth muscle was reported by Zhang et al. (1991).

Investigations on the nature of muscarinic receptors present on parietal cell membranes using binding studies and polymerase chain reaction amplification or parietal cell messenger RNA revealed the existence of only a M_3 receptor responsible for acid secretion (Kajimura et al. 1992).

Spalding et al. (2002) described a novel muscarinic agonist, AC-42, and demonstrated its selectivity for the M_1 muscarinic subtype.

The phenotypes of knockout mice or the responses lost in these animals were used to characterize the properties of the subtypes of muscarinic receptors (Birdsall et al. 2001). Champtiaux et al. (2003) investigated the subunit composition of functional nicotinic receptors in dopaminergic neurons with knockout mice.

Owicki et al. (1990, 1992), McConnell et al. (1991, 1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells were transfected with the m_1 muscarinic acetylcholine receptor. Sequential addition of increasing doses of carbachol every 2.5 min induced an increasing acidification allowing the determination of an EC_{50} value. The effect was antagonized by atropine.

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J.3.6.2

H₂-Antagonism

J.3.6.2.1

General Considerations

Three classes of histamine receptors with subtypes for the H₂ receptor have been identified by selective antagonists (Haaksma et al. 1990; Hill 1990; West et al. 1990; Clapham and Kilpatrick 1992). H₂-antagonists inhibit competitively the interaction of histamine with H₂-receptors responsible for acid secretion in the stomach. Although H₂-receptors are present in many tissues, including vascular and bronchial smooth muscle and the right atrium, H₂-antagonists interfere remarkably little with physiological functions other than gastric secretion.

The H₂ receptor was reported to be spontaneously active in transfected CHO cells (Smit et al. 1996). Based on this concept, the H₂ antagonists were reclassified; cimetidine, ranitidine and famotidine are in fact inverse agonists, whereas burimamide acts in this model system as a neutral agonist.

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J.3.6.2.2**Histamine H₂-Receptor Binding****PURPOSE AND RATIONALE**

Histamine receptors have been classified on the basis of pharmacological analysis (Hill et al. 1997). Histamine exerts its action via at least four receptor subtypes. The H₁ receptor couples mainly to G_{q/11}, thereby stimulating phospholipase C, whereas the H₂ receptor interacts with G_s to activate adenylyl cyclase. The histamine H₃ and H₄ receptors couple to G_i proteins to inhibit adenylyl cyclase, and to stimulate MAPK.

Histamine H₂-receptor binding can be determined using homogenates from guinea pig cerebral cortex and ³H-tiotidine as labeled ligand (Gajtkowski et al. 1983; Norris et al. 1984; Hill 1990). Using the polymerase chain reaction, the gene encoding the histamine H₂ receptor has been cloned (Gantz et al. 1991).

PROCEDURE**Preparation of Membranes**

Guinea pigs of either sex weighing 400–600 g are sacrificed by exsanguination and the brains rapidly removed. The cerebral cortex is dissected away from the rest of the brain and homogenized in 50 mM sodium-potassium buffer, pH 7.4, using a Potter homogenizer. The homogenate is then centrifuged at 50,000g for 10 min at 4°C. The resulting pellet is washed three times by being resuspended in phosphate buffer followed by recentrifugation. The pellet is finally resuspended in phosphate buffer, pH 7.4, at a protein concentration of 5 mg/ml.

Assay

An aliquot of 100 μl of the homogenate is incubated with 2 nM ³H-tiotidine and varying concentrations of competing test substance, in triplicate, in a total volume of 250 μl, for 30 min at room temperature. The reaction is stopped by the addition of 2 ml of ice-cold phosphate buffer and immediately filtered under reduced pressure through Whatman GF/B glass-fiber filters, followed by 3 times 3 ml washes with room temperature buffer.

Radioactivity is determined by allowing the filters to remain for at least 18 h in NE 260 scintillator (Nuclear Enterprise), followed by liquid scintillation counting.

EVALUATION

K_i values (μM) are calculated for displacement of specific H₂-binding from the relationship

$$K_i = IC_{50} / (1 + [L]K_d^{-1})$$

where IC₅₀ is the concentration of the drug required for 50% inhibition of specific binding, [L] is the concentration of ³H-tiotidine in the assay and K_d is the dissociation constant for ³H-tiotidine.

Data can be analyzed using a computer program as described by McPherson (1985).

MODIFICATIONS OF THE METHOD

Hirschfeld et al. (1992) performed photoaffinity labeling studies of the H₂ receptor using the radioactive probes [¹²⁵I]iodoaminopotentidine and its photolabile azido analogue [¹²⁵I]iodoazidopotentidine.

Martinez-Mir et al. (1990) studied the distribution of histamine H₁, H₂, and H₃ receptors in postmortem human and rhesus monkey brain by receptor autoradiography using [¹²⁵I]iodobolpyramine, [¹²⁵I]iodoaminopotentidine, and [³H](R)α-methylhistamine as ligands to label H₁, H₂, and H₃ receptors, respectively.

Traiffort et al. (1992) used [¹²⁵I]iodoaminopotentidine for pharmacological characterization and auto-radiographic localization of histamine H₂ receptors in human brain.

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J.3.6.2.3

H₂-Antagonism in Isolated Guinea Pig Right Atria

PURPOSE AND RATIONALE

H₂-antagonism can be determined in isolated guinea pig right atria which contain predominantly H₂-receptors. Compounds that inhibit the positive chronotropic effect mediated by histamine H₂-receptors in the isolated right guinea pig atrium (Reinhardt et al. 1974) can be classified as specific histamine H₂-antagonists. The test can be used as screening method for H₂-antagonists.

PROCEDURE

Male guinea pigs, e.g., Hartley strain, are sacrificed by exsanguination. The right atria are dissected and suspended at 0.7 g tension in a 25 ml organ bath with Tyrode's solution bubbled with carbogen (5% CO₂/95% O₂) at 38°C. After a stabilization period of 30 min, the contractions are recorded on a polygraph with a force-displacement transducer through a strain gauge. Cumulative concentration-response curves are obtained after sequential additions of histamine (10⁻⁷ to 10⁻⁴ M) in the absence of test drugs and the equilibration of test drugs or the standard (cimetidine).

EVALUATION

pA₂ values and relative potencies are calculated by a Schild plot (Arunlakshana and Schild 1959).

MODIFICATIONS OF THE METHOD

Hattori et al. (1990) studied the inotropic, electrophysiological and biochemical responses to histamine in rabbit papillary muscles and found evidence for coexistence of H₁- and H₂-receptors. Histamine increases force of contraction and decreases action potential duration in rabbit papillary muscles. H₂-antagonists antagonize the histamine-induced decrease in action potential duration, however, are less effective in antagonizing the increase in force of contraction produced by histamine. The positive inotropic response to his-

tamine is abolished by sequential addition of a H₁-antagonist.

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J.3.6.2.4

H₂-Antagonism in Isolated Rat Uterus

PURPOSE AND RATIONALE

Histamine inhibits spontaneous and electrically stimulated contractions of rat uterus horns. This effect can be antagonized by H₂-, but not by H₁-antagonists.

PROCEDURE

Adult, virgin Wistar rats weighing 180–200 g are used. By microscopic examination of vaginal smears, the stage of the estrus cycle is determined. Only animals in natural proestrus or metestrus are chosen. After sacrifice, the two uterus horns are removed and placed in modified de Jalon solution at room temperature and cleaned of mesenteric fat and connective tissue. Paired preparations from the same animal are used in parallel experiments. Two segments, about 2 cm in length, are taken from the ovarian end of the uterus horns and mounted separately on Perspex organ holders between two steel electrodes arranged at the end of the muscle. The organs are immediately superfused with modified de Jalon solution at 35°C at a rate of 2–3 ml/min being continuously gassed with air. Basal muscle tension is maintained at 0.5 g. The contractions of the preparations are recorded isometrically with a force-displacement transducer linked to a multipen recorder. The intrinsic rhythm of spontaneous and regular con-

tractions appears within 5–10 min after mounting the tissues in their holders.

Organs showing a frequency of only one contraction every two min or less are electrically stimulated with square wave impulses of 2 ms duration for 1 s every 1 min at 10 V and 80 Hz with a suitable stimulator. The organs are linked via a four-way stopcock to a set of reservoirs of modified de Jalon solution containing different concentrations of agonist and test substance. The preparations are superfused for 20–30 min with nutrient solution until mechanical activity of the uterus horns is stabilized in frequency and contraction height before addition of drugs.

The time interval between application of drugs is 15–20 min, the time of contact of the drugs with the uterus segments is about 4–5 min, being interrupted as soon as the contractions of the muscles reach a minimum. Logarithmic dose-response curves for histamine are constructed from mean effects of single doses, taking the average amplitude of 5 contractions immediately preceding the addition of histamine as 100% control activity. In experiments involving antagonists, a standard dose of histamine is used giving a response within the linear region of the log dose-response curve and is set as 100%. Reduction of histamine-induced inhibition is studied by superfusing increasing concentrations of antagonists in addition to the standard dose of histamine.

EVALUATION

Results are expressed in percentage of the reversal of initial depression of contraction height after histamine and plotted against $-\log$ mol/l concentration of the antagonist. pA_2 values are calculated according to Ariëns and van Rossum (1957).

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J.3.6.2.5

Activity at Histamine H_1 - and H_2 -Receptors In Vivo

PURPOSE AND RATIONALE

Owen and Pipkin (1985) described a technique that allows simultaneous and quantitative assay of the action of agonists and antagonists at histamine H_1 - and H_2 -receptors in anesthetized guinea pigs. The principle

of the technique is based on H_1 -receptor caused bronchoconstriction and H_2 -receptor caused tachycardia.

PROCEDURE

Guinea pigs of either sex weighing about 500 g are anesthetized with 90 mg/kg sodium pentobarbitone i.p. The trachea is cannulated to permit artificial respiration. Blood pressure is measured from a catheter tied into one carotid artery. Catheters are tied into one jugular and one femoral vein for the administration of drugs.

Airways resistance is measured using a modification of the Konzett–Rössler technique. By means of a small animal respiration pump, the lungs are inflated at a rate of 40 breaths/min. The inflow arm of the circuit includes a side-arm, the outlet of which is placed below 12 cm of H_2O . The volume of air used in the study has to be that which fills the lungs at a pressure of 12 cm H_2O , selected by adjusting the volume until no air escapes through the H_2O trap each time the lungs are filled. The side-arm is then clamped, and the animal is respired with the selected volume for the duration of the study. Airways resistance to inflow is measured using a pressure transducer connected to a second arm of the inflow circuit. Resistance is proportional to the maximum pressure required to inflate the lungs. Inflow pressure is registered on an electronic recorder.

Heart rate is measured from the blood pressure pulse using an instantaneous rate meter and is registered on an electronic recorder.

Intravenous injection of histamine causes simultaneously bronchoconstriction and tachycardia. The threshold dose needed to cause tachycardia may be less than that for bronchoconstriction, but both responses will be apparent over the dose range 1×10^{-8} to 5×10^{-7} mol/kg.

EVALUATION

Agonists

Dose-response curves for agonists compared to the dose-response curve for histamine for both parameters, bronchoconstriction and tachycardia, allow the calculation of potency ratios. Specific H_1 -receptor agonists are more potent to cause bronchoconstriction than tachycardia, whereas H_2 -agonists provoke tachycardia but are less active or inactive causing bronchoconstriction.

Antagonists

To evaluate antagonists, various doses of the test compound are injected i.v. and dose-response curves of

histamine are established for both parameters to be compared with the dose-response curve of histamine without pretreatment. From the ratios of shift to the right dose-response curves for both parameters can be established. These are similar for mixed antagonists, but a definitively higher potency is shown for H₁-antagonists in bronchoconstriction and a higher potency for H₂-antagonists in tachycardia.

REFERENCES AND FURTHER READING

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J.3.6.2.6

Inhibition of Histamine Stimulated Adenylate Cyclase from Gastric Mucosa

PURPOSE AND RATIONALE

The *in vitro* potencies of H₂-antagonists can be evaluated using determinations of adenylate cyclase activity in membrane preparations of guinea pig mucosa after stimulation with histamine.

PROCEDURE

Preparation of Membranes

Guinea pigs of either sex weighing 400–600 g are sacrificed. The fundic portion of the stomach is rapidly removed. Food particles are washed away with ice-cold 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA and 0.25 M sucrose (homogenization buffer). The tissue is stretched, mucosal side up, on a glass Petri dish supported on ice and the mucosal layer is scraped off the muscle layer using a scalpel blade. The mucosal scrapings are transferred to 10 ml ice-cold homogenization buffer in a glass homogenization tube, and homogenized using an Ultra-Turrax homogenizer followed by four strokes using a Potter Teflon homogenizer. The resulting suspension is centrifuged at 700 g for 10 min at 4°C. The supernatant is discarded, and the pellet resuspended in 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA followed by a further period of centrifugation. The resulting pellet is finally resuspended in Tris/EDTA buffer at a protein concentration of 2.4 mg/ml as determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Stimulation of Adenylate Cyclase

Adenylate cyclase activity is measured according to Hegstrand et al. (1976). Aliquots (120 µg) of fundic mucosal homogenate are incubated in

100 mM Tris buffer, pH 7.8, containing 0.6 mM EGTA (ethylene-glycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid), 1 mM IBMX (isobutyl-methyl xanthine), 2 mM MgCl₂, 0.1 mM GTP (guanosine triphosphate), histamine at various concentrations between 0.1 µM and 100 µM and the compound under investigation at varying concentrations between 0.1 µM and 1 mM for 10 min at 0°C. The reaction is initiated by the addition of 1 mM ATP followed by a 10 min incubation at 30°C. The reaction is terminated by placing the assay tubes in a boiling water bath for 3 min. After cooling, 40 mg of Alumina 90 (E. Merck, 70–230 mesh) are added to each tube prior to mixing and centrifugation at 700 g for 15 min at 4°C.

Assay of Cyclic AMP

The cyclic AMP content of each sample is determined according to Brown et al. (1971) using ³H-cyclic AMP in a competitive protein binding assay. Fifty µl aliquots from the adenylate cyclase assay are incubated with 50 µl of ³H-cyclic AMP (14 nCi at a specific activity of 62 Ci/mmol) and 100 µl of previously prepared cyclic AMP binding protein at 4°C. The bound and free ³H-cyclic AMP are separated by the addition of 100 µl of charcoal reagent (2 g bovine serum albumin, 2.5 g Norit GSX charcoal in 100 ml of 50 mM Tris buffer, pH 7.4, containing 4 mM EDTA), followed by mixing and centrifugation at 1000 g for 15 min. Two hundred µl aliquots of the supernatant are decanted into scintillation vials containing 10 ml of NE 260 scintillator. Radioactivity is determined by liquid scintillation counting.

EVALUATION

Results are calculated from a standard curve using cyclic AMP as the standard. From these data IC₅₀ values (µM) are derived.

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J.3.6.3

H⁺/K⁺-ATPase (Proton Pump) Inhibition

J.3.6.3.1

General Considerations

The parietal cell of the stomach is activated by three major stimuli: histamine, acetylcholine, and gastrin. In addition to the direct action of gastrin and acetylcholine on the parietal cell, these two agents may release histamine from a histamine storage in the gastric mucosa. In this manner, histamine would act as the final mediator of acid secretion. One of the first events leading to acid secretion is a massive membrane transformation that occurs in the parietal cell. When the cell is stimulated, the tubulovesicles in the cytoplasm of the cell fuse and form an expanded secretory canaliculus in the apical membrane where the enzyme H⁺/K⁺-ATPase is located.

In this way, the ultimate mediator of acid secretion in the stomach is the proton pump H⁺/K⁺-ATPase which transports hydrogen in exchange for potassium. The rat stomach H⁺/K⁺-ATPase has been cloned by Shull and Lingrel (1986). Development of specific inhibitors of this enzyme is an approach to suppress acid secretion and ulcer formation since ulcers only exist in acidic medium.

Alderuccio et al. (1993) described an experimental autoimmune gastritis in BALB/c mice as a CD4⁺ T cell-mediated organ-specific autoimmune disease induced by neonatal thymectomy. Transgenic expression of the gastric H⁺/K⁺-ATPase β subunit specifically prevented the onset of this form of autoimmune gastritis.

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J.3.6.3.2

H⁺/K⁺-ATPase Inhibition in Membrane Vesicles of Stomach Mucosa

PURPOSE AND RATIONALE

Several H⁺/K⁺-ATPase inhibitors contain a sulfhydryl group. At lower pH-values the compounds are protonated and rearrange to a sulphenic acid and a sulphenamide, that react with sulfhydryl groups in the enzyme. Therefore, the *in vitro* assays are performed both at neutral and at acidic pH levels.

PROCEDURE

Membrane vesicles containing H⁺/K⁺-ATPase are prepared from pig stomachs obtained from the local slaughter house (Ljungstrom et al. 1984). Pigs are fasted overnight before slaughter. The gastric mucosa of four stomachs is rinsed with cold saturated NaCl solution for 3–5 min. The superficial cells, cell debris plus the mucus are wiped off with the edge of a plastic ruler and with paper towels. The mucosa is scraped off. About 100 g scrapings are divided into portions of 10 g and homogenized in 0.25 M sucrose with seven strokes in a Potter-Elvehjem Teflon-glass homogenizer. The total volume is 600 ml which is centrifuged at 20,000 g for 40 min. The pellet is discarded. The supernatant is centrifuged at 75,000 g for 1 h. The resulting microsomal pellet is homogenized in 30 ml 0.25 M sucrose.

Aliquots of 15 ml are transferred to 100 ml centrifuge tubes and layered on top of step gradients, from the bottom comprising 25 ml 37% sucrose (w/v) and 45 ml 7.5% Ficoll (w/v) in 0.25 M sucrose. The tubes are centrifuged at 75,000 g for 1 h in a 6 × 100 ml ME angle rotor at 4°C. The gradient is then fractionated by pumping Fluoroinert 70 through a narrow tubing in a fractionating cap down to the bottom of the tube. Fractions are collected from top through a center hole in the fractionating cap. The yield of vesicles in a typical preparation is about 50–75 mg protein. In order to maintain a stable vesicular structure for a long period of time, the vesicles are frozen at –70°C under nitrogen. They can then be kept for several months without decrease of H⁺/K⁺-ATPase activity.

The ATPase activity is measured at 37°C as the release of inorganic phosphate (P_i) from ATP. The test drug and the standard (omeprazole) are pre-incubated in concentrations of 0.01 to 100.0 μM in enzyme containing buffers in parallel at pH 6.0 and 7.4 for 30 min at 37°C. Then, the medium of pH 6.0 is adjusted with

HEPES/Tris buffer to pH 7.4. The enzyme reaction is started by addition of nigericin and Tris/ATP. The total reaction volume is 1 ml, containing 20 µg vesicular protein, 4 mM MgCl₂, 10 mM KCl, 20 µM nigericin, 2 mM Tris-ATP, 10 mM HEPES and additionally 2 mM Pipes for the pre-incubation medium at pH 6.0.

After 4 min at 37°C, the reaction is stopped by the addition of 10 ml of 50% trichloroacetic acid. The denatured protein is spun down and the P_i content is determined according to LeBel et al. (1978) based on the reduction of a phosphomolybdate complex by p-methylaminophenol sulfate in a copper acetate buffer or according to Carter and Karl (1982) based on the reaction of phosphomolybdate with the basic dye malachite green.

EVALUATION

IC₅₀ values are calculated by probit analysis, whereby 0% corresponds to 4 mM Mg²⁺-dependent and 100% to 4 mM Mg²⁺ plus 10 mM K⁺-dependent ATP hydrolysis. IC₅₀ values of the test compound at different pH values are compared with IC₅₀ values of the standard. Statistical differences (*p* < 0.05) are calculated by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Proton transport in gastric vesicles can be measured by acridine orange fluorescence quenching (Lee and Forte 1978; Beil et al. 1990). Membrane protein (0.12 mg) is incubated at 37°C in a volume of 2 ml containing: 10 mM Pipes/Tris buffer, pH 7.0 in the presence of 150 mM KCl, 2 mM MgCl₂, 2 mM ATP and 10 µM acridine orange. The pump reaction is started by the addition of valinomycin (ionophore for K⁺). The decrease of fluorescence is studied at 530 nm as a measure for the intravesicular proton uptake.

CRITICAL ASSESSMENT OF THE METHOD

The pre-incubation period at the lowest possible pH of about 6 is used to initiate the acidic conversion of the test compound into its active principle. This reflects more the chemical instability of the test compound at neutral pH values than its effect during conditions of much higher acidity within the secretory canaliculus of the parietal cell during acid secretion. Many chemically labile inhibitors are therefore very active in this test system. However, they do not cause an inhibition in more complex test systems and, therefore, are without any practical usefulness (Lindberg et al. 1990). Proton transport studies in gastric vesicles, using the acridine orange fluorescence quenching technique, where a pH gradient similar to *in vivo* condi-

tions is formed (Lee and Forte 1978; Beil et al. 1990) are more suitable for studying the mechanism of action, acid-conversion, and structure-activity relationship of K⁺/H⁺-ATPase inhibitors (Herling and Weidmann 1994).

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J.3.6.3.3

Effect of H⁺/K⁺-ATPase Inhibitors on Serum Gastrin Levels

PURPOSE AND RATIONALE

It is known from the H⁺/K⁺-ATPase inhibitor omeprazole that the total acid blockade initiates a gastric antral feed back mechanism resulting in an excessive hypergastrinaemia (Arnold et al. 1986; Creutzfeldt et al. 1986; Larsson et al. 1986) which is believed to cause diffuse endocrine cell hyperplasia, characterized as carcinoids, in the gastric corpus after 2 years of treatment in the rat (Ekman et al. 1985).

PROCEDURE

Groups of 10–15 female Wistar rats weighing 90–110 g are treated daily for 10 weeks with omeprazole (10 or 30 mg/kg p.o.) or the test compound or serve as controls. The compounds are suspended in potato starch mucilage (20 mg/ml) and administered in a volume of 2 ml/kg. On days 1–3, the rats receive the H⁺/K⁺-ATPase inhibitors by intraperitoneal injection in order to cause gastric acid inhibition and therefore to reduce the gastric acid degradation of subsequent oral doses. After treatment for 2, 4, 7, and 10 weeks, blood samples are collected under ether anesthesia by retroorbital puncture. Gastrin is determined by radioimmunoassay using a commercially available kit, e. g. Gastrin RIAKit II; Dainabot Co., Ltd. At the end of the study of 10 weeks, the animals are studied for their gastric acid output using the pylorus ligation (Shay technique).

EVALUATION

Serum gastrin levels are determined as pg/ml. Statistical differences ($p < 0.05$) are calculated using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Katz et al. (1987) described a five-day test to predict the long-term effects of gastric antisecretory agents on serum gastrin in rats.

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of gastric H⁺ secretion (Berglindh and öbrink 1967; Berglindh et al. 1967; Sack and Spenny 1982; Sewing et al. 1983; Herling et al. 1987, 1988, 1990). In isolated gastric glands and parietal cells, H⁺ secretion cannot be directly measured by titration; therefore, accumulation of weak bases such as aminopyrine is used as an indirect probe of H⁺ secretion. Moreover, glandular oxygen consumption can be measured with the Warburg technique.

PROCEDURE**Preparation of Gastric Glands**

Rabbits are anesthetized with 40 mg/kg pentobarbital i.v. The abdomen is opened and the aorta is cannulated in a retrograde direction. Five ml of a heparin solution (250 IU/ml) are injected with force through the cannula. After one min the rabbit is bled through the cannula and a ligature is placed around the mesenteric vessels. The chest is quickly opened and the thoracic aorta clamped. Phosphate buffered saline solution (containing 149.6 mM NaCl, 3 mM K₂HPO₄, 0.64 mM NaH₂PO₄, pH 7.3) at 37°C is pumped through the aorta, whereby the portal vein is opened to allow free outflow of the perfusate. By this procedure most of the solution is forced through the mesenteric vessels. The perfusion pressure, as measured proximal to the cannula, can be up to 600 mm Hg.

The stomach appears totally exsanguinated after perfusion with about 500 ml phosphate buffered saline solution and is then removed, cut open along the lesser curvature, and emptied. The cardiac and antral regions are discarded. The corpus is rinsed several times with phosphate buffer solution and finally blotted with filter paper, whereby the remaining gastric content as well as some surface epithelial cells are removed. By blunt dissection the mucosa can easily be separated from the muscular and submuscular layers.

The mucosa is then minced into small pieces with a pair of scissors. The pieces are washed twice in warm oxygenated phosphate buffer solution and transferred to a 200 ml flask with 50 ml of a freshly prepared collagenase-enzyme solution containing 1 mg/ml collagenase (type I, Sigma), 1 mg/ml rabbit albumin (Sigma), 2 mg/ml glucose in 130 mM NaCl, 10 mM NaHCO₃, 3 mM NaH₂PO₄, 3 mM Na₂HPO₄, 3 mM K₂HPO₄, 2 mM MgSO₄, 1 mM CaCl₂, and 10 mg/L phenol-red at pH 7.4. The flask is gassed with 100% oxygen, sealed, and kept in a 37°C water bath under gently stirring with a magnet for 90 min. The cloudy suspension containing the separated glands and some cells is filtered through a nylon mesh into 15 ml test tubes with conical bottoms. The glands rapidly sediment to the

J.3.6.3.4**(¹⁴C)-Aminopyrine Uptake and Oxygen Consumption in Isolated Rabbit Gastric Glands****PURPOSE AND RATIONALE**

Isolated gastric glands from rabbits and other species can be used in studying the control of mechanism

bottom while the free cells remain in the solution. In this way centrifugation can be avoided and the glands can easily be washed free from isolated cells and collagenase by three washings with phosphate buffer at room temperature.

¹⁴C-Aminopyrine Accumulation in Gastric Glands

The ability of gastric glands to form acid is measured based on aminopyrine accumulation. The glands are diluted to a final concentration of 2–4 mg dry weight/ml in a medium containing 100.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH₂PO₄, 1 mM Na₂HPO₄, 1.0 mM CaCl₂, 1.5 mM MgCl₂, 20.0 mM NaHCO₃, 20.0 mM HEPES, 2 g/ml glucose, 1 mg/ml rabbit albumin, adjusted with 1 M Tris to pH 7.4. Samples of 1 ml gland suspension are equilibrated in 1 ml medium containing 0.1 μCi/ml [¹⁴C]aminopyrine at 37°C in a shaking water bath together with the agent to be tested. After 20 min either histamine or dibutyl-cyclic-AMP is added, followed by a 30 min incubation period for histamine and a 45 min incubation period for dibutyl-cyclic-AMP.

The glands are then separated from the medium by brief centrifugation. The supernatant is withdrawn, the pellets are dried at 80°C for 50 min, weighed and dissolved in 200 μl 1 M NaOH. Aliquots of the supernatant and the digested gland pellet are examined in a liquid scintillation counter. The ratio of intraglandular to extraglandular radioactivity is calculated. All determinations are made in triplicate.

Respiratory Studies

Glandular oxygen consumption is measured at 37°C using a Warburg respirometer and air as the gas phase. The 15 ml flasks with a central well 20% containing KOH solution on a filter paper as CO₂ adsorber are filled with 1 ml medium containing 100.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH₂PO₄, 1 mM Na₂HPO₄, 1.0 mM CaCl₂, 1.5 mM MgCl₂, 20.0 mM NaHCO₃, 20.0 mM HEPES, 2 g/ml glucose, 1 mg/ml rabbit albumin, adjusted with 1 M Tris to pH 7.4 and 1 ml gland suspension. After 20 min equilibration, the test compound and dbcAMP are added and the oxygen consumption is measured at 15 min intervals for the following 45 min. The recorded oxygen consumption is corrected according to the following formula:

$$\text{O}_2 \text{ consumption} = \text{O}_2 \text{ recorded} \times K$$

where

- $K = 273 \times (Pb/t + 273) \times 760$
- t = ambient temperature in °C around the manometers

- Pb = Atmospheric pressure (mm Hg)

The respiratory activity is expressed in μl O₂ consumed per mg dry weight and time.

EVALUATION

IC_{50} values are calculated by probit analysis. Statistical differences ($P < 0.05$) are assessed by Student's t -test, n = the number of different gland preparations.

CRITICAL ASSESSMENT OF THE METHOD

Studies on (¹⁴C)-aminopyrine uptake and oxygen consumption in isolated rabbit gastric glands have become a valuable approach for studying the effect of various H₂-receptor antagonists and proton pump inhibitors. When studying gastric acid production with the ¹⁴C-AP accumulation technique, the addition of basic drugs can be problematic (Fryklund and Wallmark 1986). The basic nature of a test compound can compete with accumulation of ¹⁴C-AP. Nevertheless, it is generally accepted that oxygen consumption of gastric glands correlates well with acid formation (Berglindh et al. 1976).

MODIFICATIONS OF THE METHOD

Soll (1978) studied the actions of secretagogues on oxygen uptake by isolated mammalian parietal cells. Parietal cells were prepared from the stomach of dogs by collagenase digestion and counterflow centrifugation. Oxygen consumption was determined by polarography. Isobutyl methyl xanthine (IMX), carbamylcholine, histamine, and gastrin each independently stimulated oxygen uptake. The specificity of these responses was tested by use of an H₂-histamine receptor antagonist or atropine as anticholinergic agent.

Stoll (1980) investigated the [¹⁴C]aminopyrine accumulation in isolated *canine* parietal cells when treated with histamine, gastrin and carbachol and the displacement by cimetidine or atropine.

Sewing et al. (1983, 1986) studied the effect of several benzimidazole derivatives on isolated and enriched guinea pig parietal cells using the ¹⁴C-aminopyrine accumulation.

Scheppe et al. (1994) determined the effects of exendin-4, a peptide from *Heloderma suspectum venom*, and exendin-(9–39)NH₂ on [¹⁴C]aminopyrine accumulation in isolated rat parietal cells and compared these with those of glucagon-like peptide-1-(7–36)NH₂.

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J.3.6.3.5

Gastric Mucosal Blood Flow

PURPOSE AND RATIONALE

Hydrogen gas clearance has been used to measure blood flow in the basal portion of gastric mucosa in anesthetized rats (Leung et al. 1984, 1986; Pique et al. 1988).

PROCEDURE

Male Sprague Dawley rats are anesthetized with 1.5 g/kg of urethane subcutaneously. A tracheotomy is performed and a PE-250 tubing is inserted into the trachea to facilitate spontaneous breathing and for admin-

istration of 3% hydrogen in air. The right carotid artery is cannulated for blood pressure monitoring. A mid-line laparotomy is then performed and the stomach exteriorized. Through an incision in the forestomach the gastric contents are gently washed out with physiological saline. A double-lumen cannula (outer: Tygon with a diameter of 7 mm; inner: polyethylene with a diameter of 2 mm) are inserted into the stomach and secured by a ligature at the forestomach. The pylorus is ligated and a 0.9% sodium chloride solution is infused through the inner cannula at a rate of 0.8 ml/min and drained from the outer tubing. The gastric effluent is collected at 15-min intervals. Acid output (in micro-equivalents per min) is determined in the perfusate with 0.2 N NaOH with an automatic titrator.

An incision in the serosa and the muscularis externa is made in the gastric wall of the corpus, exposing 3–4 mm of the submucosa. Through this hole a platinum electrode is placed in contact with the exposed basal portion of the mucosa. The electrode is made with a ring of platinum wire, 125 μm in diameter, wound around a glass capillary tube and held in place inside a Teflon tube by epoxy. A Ag-AgCl reference electrode is placed inside the peritoneal cavity. The laparotomy incision is covered by Parafilm to minimize evaporation.

One femoral vein is cannulated for the infusion of saline or drugs. The rat is kept warm with a heat lamp to maintain rectal temperature at 37°C.

Hydrogen Gas Clearance Technique

Current is generated at the surface of a platinum electrode by oxidation of molecular hydrogen to hydrogen ions and electrons. This current, which is measured using a polarographic and amplifying unit, is proportional to the hydrogen tension gradient at the surface of the platinum electrode. When the experimental animal breathes 3% hydrogen in air, the current tracing, graphed on a recorder, gradually rises and reaches a plateau as the tissue adjacent to the electrode is saturated with hydrogen. After the external hydrogen source is removed, the current tracing gradually falls because of removal of tissue hydrogen by blood flow. The rate of dissipation of hydrogen estimates the tissue blood flow (Aukland et al. 1964).

The exponential decrease of the hydrogen gas clearing curves is evaluated by a computer program (Livingstone et al. 1986). Current from the platinum electrode is passed through an ADALAB analog-to-digital converter and discrete digitized values are sampled every 5 s. Blood flow is determined by an Newtonian-Gaussian nonlinear iterative regression program by

means of a biexponential formula:

$$f(x) = A + B e^{-k_1 t(t-T)} + B e^{-k_2 t(t-T)},$$

where $f(x)$ is the electrode current, A is the baseline current, B is the initial current, e is the base of natural logarithms, k_1 is the rate constant for the fast component, k_2 is the rate constant for the slow component, t is the time at which the current is observed, and T is the time when hydrogen gas was removed.

Mucosal blood flow is expressed in milliliters per minute per 100 g of tissue.

Inhibition of Acid Secretion

Intravenous saline is infused during the first 45 min of the study. After this period, an infusion of 80 $\mu\text{g}/\text{kg} \cdot \text{h}$ of pentagastrin is administered for 135 min. During the last 75 min an intravenous infusion of the inhibitor or vehicle is administered simultaneously with pentagastrin. Corpus mucosal blood flow measurements are obtained during the resting period, 45 min after start for the pentagastrin infusion, and during the last 15 min of combined infusion of pentagastrin and inhibitor. Acid output is measured at 15 min intervals throughout the experiments.

EVALUATION

All data are expressed as mean \pm standard error. The data are analyzed using a paired t -test for comparison of basal versus stimulated condition within the same animal, analysis of variance with contrasts, and linear and polynomial regression analysis for comparison between animals in different groups. A probability level of $P < 0.05$ is considered significant.

MODIFICATION OF THE METHOD

The hydrogen gas clearance technique has been used by many authors in experimental gastroenterology, e.g.: Hirose et al. (1991), Holzer and Guth (1991), Lippe and Holzer (1992), Pique et al. (1992), Tsukamoto et al. (1992), Lazaratos et al. (1993), Petho et al. (1994), Tanaka and Guth (1994), Goldin et al. (1996), Hisanaga et al. (1996), Doi et al. (1998), Heinemann et al. (1999).

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J.3.7

Anti-Ulcer Activity

J.3.7.1

Pylorus Ligation in Rats (SHAY Rat)

PURPOSE AND RATIONALE

A simple and reliable method for production of gastric ulceration in the rat based on ligation of the pylorus has

been published by Shay et al. (1945). The ulceration is caused by accumulation of acidic gastric juice in the stomach.

PROCEDURE

Female Wistar rats weighing 150–170 g are starved for 48 h having access to drinking water ad libitum. During this time they are housed single in cages with raised bottoms of wide wire mesh in order to avoid cannibalism and coprophagy. Ten animals are used per dose and as controls. Under ether anesthesia a mid-line abdominal incision is made. The pylorus is ligated, care being exercised that neither damage to the blood supply nor traction on the pylorus occurs. Grasping the stomach with instruments is to be meticulously avoided, else ulceration will invariably develop at such points. The abdominal wall is closed by sutures. The test compounds are given either orally by gavage or injected subcutaneously.

The animals are placed for 19 h in plastic cylinders with an inner diameter of 45 mm being closed on both ends by wire mesh. Afterwards, the animals are sacrificed in CO₂ anesthesia. The abdomen is opened and a ligature is placed around the esophagus close to the diaphragm. The stomach is removed, and the contents are drained in a centrifuge tube. Along the greater curvature the stomach is opened and pinned on a cork plate. The mucosa is examined with a stereomicroscope. In the rat, the upper two fifths of the stomach form the rumen with squamous epithelium and possess little protective mechanisms against the corrosive action of gastric juice. Below a limiting ridge, in the glandular portion of the stomach, the protective mechanisms are better in the mucosa of the medium two fifths of the stomach than in the lowest part, forming the antrum. Therefore, lesions occur mainly in the rumen and in the antrum. The number of ulcers is noted and the severity recorded with the following scores:

- 0 = no ulcer
- 1 = superficial ulcers
- 2 = deep ulcers
- 3 = perforation.

The volume of the gastric content is measured. After centrifugation, acidity is determined by titration with 0.1 n NaOH.

EVALUATION

An ulcer index U_I is calculated:

$$U_I = U_N + U_S + U_P \times 10^{-1}$$

- U_N = average of number of ulcers per animal
- U_S = average of severity score
- U_P = percentage of animals with ulcers

Ulcer index and acidity of the gastric content of treated animals are compared with controls. Using various doses, dose-response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0% corresponds to no and 100% to maximal stimulated gastric acid output.

CRITICAL ASSESSMENT OF THE METHOD

The “Shay-rat” has been proven to be a valuable tool to evaluate anti-ulcer drugs with various mechanisms of action.

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J.3.7.2

Indomethacin Induced Ulcers in Rats

PURPOSE AND RATIONALE

Nonsteroidal anti-inflammatory agents, like indomethacin and acetyl-salicylic acid, induce gastric lesions in man and in experimental animals by inhibition of gastric cyclo-oxygenase resulting in less formation of prostacyclin, the predominant prostanoid produced in the gastric mucosa.

PROCEDURE

Groups of 8–10 Wistar rats weighing 150–200 g are used. The test drugs are administered orally in 0.1% Tween 80 solution 10 min prior to oral indomethacin in a dose of 20 mg/kg (4 mg/ml dissolved in 0.1% Tween 80 solution). Six hours later, the rats are sacrificed in CO₂ anesthesia and their stomachs removed. Formol-

saline (2% v/v) is then injected into the totally ligated stomachs for storage overnight. The next day, the stomachs are opened along the greater curvature, then washed in warm water, and examined under a 3-fold magnifier. The lengths of the longest diameters of the lesions are measured and summated to give a total lesion score (in mm) for each animal, the mean count for each group being calculated.

EVALUATION

The mean score in control rats is about 25 (range 20–28). Inhibition of the lesion production is expressed as percentage value.

MODIFICATION OF THE METHOD

Dose- and time dependency of the ulcerogenic action of indomethacin were studied by Djahanguiri (1969).

Instead of indomethacin, gastric lesions can be induced by intravenous or oral doses of aspirin which can be prevented by exogenous PGE₂ or PGI₂ (Konturek et al. 1981). Furthermore, reserpine at a dose of 8 mg/kg i.p., or cysteamine hydrochloride at a dose of 400 mg/kg s.c. was given in order to induce ulcers in rats (Tarutani et al. 1985).

Kitajima et al. (1993) studied the role of endothelin and platelet-activating factor in indomethacin-induced gastric mucosal injury in rats. Four hours after subcutaneous injection of 25 mg/kg indomethacin, the rats were sacrificed after ether anesthesia, and the stomach was removed. The stomach was filled with 1.5 ml of 2% buffered formalin for 10 min and then opened along the greater curvature. The total length of the lesions was measured.

Wallace et al. (1989) studied the ulcerogenic activity of endothelin in indomethacin pretreated rats using an *ex vivo* gastric chamber.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement and recording gastric potential difference in the rat.

CRITICAL ASSESSMENT OF THE METHOD

According to West (1982) the cold stress induced ulcer formation, but not the indomethacin- or aspirin-induced ulcers are inhibited by H₂-receptor antagonists, whereas other authors reported protective effects of H₂-receptor antagonists under these conditions (Tarutani et al. 1985).

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J.3.7.3

Ethanol Induced Mucosal Damage in Rats (Cytoprotective Activity)

PURPOSE AND RATIONALE

Intragastric application of absolute ethanol is a reproducible method to produce gastric lesions in experimental animals (Robert et al. 1979; Szabo et al. 1981). These lesions can be at least partially inhibited by various drugs, such as some prostaglandins. The protective effect against various irritants has been called cytoprotective activity (Robert 1979; Robert et al. 1979). The method has been modified by several authors. Witt et al. (1985) described a method to objectively quantify the extent of ethanol-induced gastric lesions utilizing a transmission densitometer to measure the optical density of the photographic negative of the stomach mucosa.

PROCEDURE

Male Wistar rats weighing 250–300 g are deprived of food 18 h prior to the experiment but are allowed free access to water. During this time they are kept in restraining cages to prevent coprophagy. The rats are administered either the appropriate vehicle or the cytoprotective drug, e. g. a prostanoid, intragastrally 30 min prior to administration of 1 ml absolute ethanol. Untreated animals are included as controls. One hour after administration of ethanol, the animals are euthanized with CO₂, the stomachs are excised, cut along

the greater curvature, and gently rinsed under tap water. The stomachs are stretched on a piece of foam core mat, mucosal site up.

The subjective scores of the treated tissues are recorded; the graded response is reflecting the least (0) to most (3) damage. A circular full thickness area, about 13 mm in diameter, is cut with a cork borer from each lobe of the fundus just below the ridge dividing the glandular from the non-glandular portion of the stomach. A Plexiglas template (19 × 14 × 0.3 cm), burnished on one side with emery cloth, and with four rows with six holes 13 mm in diameter is placed on a sheet of clear glass, burnished side up, and bound to the glass with photographic tape along the periphery. The excised pairs of tissue from each stomach are placed into the holes of the template.

Pairs of tissue from each stomach are examined to minimize sampling errors. The template is positioned on a rectangular central open area of an Aristo Model T-16 cold cathode transilluminator (38 × 38 cm) containing a W-45 blue-white lamp. A camera is mounted on a copy stand directly above the template. Photographs are taken, the film processed in a standard manner and a contact sheet is made from the negatives. A light transmission densitometer (e. g. MacBeth model TD-501) is used to evaluate the negatives. The optical density of the test tissues is determined by placing each area of the negative in sequence over the aperture through which the light is transmitted. The optical density is displayed on a digital read out and recorded. Hemorrhagic or damaged areas appear bright on the negative, whereas undamaged tissue appears dark. Hence, lower optical density values are indicative of damage while higher optical densities are associated with little, or, as in the case of control, no damage.

EVALUATION

The significance of differences in optical density between control and ethanol-treated tissue is evaluated by nonpaired single-tail Student's *t*-test.

MODIFICATIONS OF THE METHOD

Cytoprotection by prostaglandins was studied in rats by prevention of gastric necrosis produced by various agents such as alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury (Robert et al. 1979) and against gastric injury produced by nonsteroidal anti-inflammatory compounds (Robert 1979; Franzone et al. 1988). The animals are fasted 48 h prior to the experiment and placed 18 h before the administration of drugs into plastic tubes to prevent coprophagy. Fifteen min af-

ter application of the test drug, the animals are given 1 ml of the irritant orally. After an additional hour, the animals are sacrificed, the stomachs removed and immediately opened along the greater curvature. Lesions are counted and scored (0 = no lesion; 1 = mild lesions; 2 = severe lesions; 4 = necrosis).

Starrett et al. (1989) employed 3.0 ml/kg ethyl alcohol (100%) or 3.0 ml/kg 0.75 N HCl as necrotizing agent.

Borella et al. (1989) studied the cytoprotective and anti-ulcer activities of the anorganic antacid Magaldrate in the rat using absolute ethanol as irritant.

Masuda et al. (1993) investigated the role of endogenous endothelin in the pathogenesis of ethanol-induced gastric mucosal injury in rats.

CRITICAL ASSESSMENT OF THE METHOD

Several prostaglandins provide cytoprotection, particularly in rats, in a dose-range which has no anti-secretory activity. However, clinical experience with prostaglandins showed that ulcer healing is only achieved at anti-secretory doses (Lindberg et al. 1990). Therefore, it seems very likely that the cytoprotective property of a compound in rats has very limited relevance to prediction of its ulcer healing potential in humans if cytoprotection is really separated from its antisecretory potential (Herling and Weidmann 1994).

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J.3.7.4

Subacute Gastric Ulcer in Rats

PURPOSE AND RATIONALE

Ezer (1988) described a method for producing standard subacute gastric ulcers in rats and for the quantitative evaluation of the healing process.

PROCEDURE

Female Wistar rats weighing 120–150 g are fasted for 24 h having access to water at libitum in cages with wire sieves at the bottom. The rats are anesthetized with ether and a polyethylene catheter including a fine steel wire with a needle tip (1.2 mm diameter) at the lower end is orally inserted into the stomach. After the cannula reaches the gastric wall, the upper end of the steel wire is pressed in a definitive manner, so as to puncture the gastric wall. Each rat is kept in the same position during the intervention in order to localize the puncture at nearly the same region of the glandular part of the stomach. The test substances are administered orally, 30 min or 24 h after puncture. Free access to food and water is provided from 2 h up to the end of the experiment. Each group consists of 8–15 rats.

The animals are sacrificed by overdose of ether at definitive time intervals after puncture. The stomach is dissected and opened along the lesser curvature, extensively rinsed in tap water and fixed to the end of a polyethylene tube of 10 mm diameter (plastic tip of an automatic pipette) in a position with the punched ulcer in the center. The end of the tube with the gastric wall is suspended in a beaker containing physiological saline, and the pressure in the tube is gradually increased with a valved rubber ball connected to the other end of the tube. The third part of the system is a tonometer calibrated up to 1 bar. The value of tension at which bubbles appear at the ulcerous gastric wall is noted. This value is termed as tensile strength and can be expressed in mm Hg.

EVALUATION

The extent of the healing of gastric ulcers can be characterized by the healing rate (*HR*) according the fol-

lowing equation:

$$HR = (A - B)/C \text{ (mmHg/h)}$$

with

- *A* = tensile strength (mm Hg) at *C* time-point after puncture
- *B* = tensile strength 30 min after puncture (the average value is 143 mm Hg)
- *C* = time course (h) of the experiment.

Anti-ulcer drugs, such as H₂ antagonists, significantly increase the healing rate, which is decreased by non-steroidal anti-inflammatory drugs.

CRITICAL ASSESSMENT OF THE METHOD

Similarly to the method of Takagi et al. (1969) who injected 50 µl of acetic acid into the stomach wall (Szeleenyi et al. 1982), the method of Ezer (1988) allows to judge the time course of healing of the ulcers.

MODIFICATIONS OF THE METHOD

Okabe and Pfeiffer (1972) induced chronic gastric ulcer in rats by temporary instillation of acetic acid. In pentobarbital anesthesia, a cylindrical glass tube of 6 mm in diameter was tightly placed upon the anterior serosal surface of the glandular portion of the stomach one cm away from the pyloric end. A dose of 0.06 ml/animal of 50% acetic acid was instilled into the tube and allowed to remain one min on the gastric wall. After removal of the acid solution, the abdomen was closed in two layers and the animals brought back to their cages and fed normally. Test drugs were given orally on day 1 twice daily, 4 h after application of acetic acid and continued up to 10 days after induction of ulcer. The animals were sacrificed after 18 h of the last dose to assess ulcer size and healing. Ulcer index was calculated upon the product of length and width of ulcers.

Karmeli et al. (1996) induced gastric mucosal erosions in rats by addition of 0.1% iodoacetamide to the drinking water. The animals were sacrificed after various time intervals, the stomach was resected, washed, lesion area assessed, and mucosal inflammatory mediators determined. Myeloperoxidase was increased and nitric oxide synthase activity decreased. The damage induced by iodoacetamide was significantly ameliorated by treatment with a free radical scavenger, (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, TEMPOL).

Piqueras et al. (2003) described gastric hypersecretion in **mice** due to mast cell activation associated with

mild gastritis induced by 0.1% iodoacetamide administered intragastrally and added to the drinking water for a 6-day period.

Marchetti et al. (1995) and Konturek et al. (1999) described a mouse model of *Helicobacter pylori* infection. Gastric function and healing of chronic acetic acid-induced ulcers in BALB/c mice were studied after inoculation with CagA and VacA positive (type I) or CagA and VacA negative (type II) *Helicobacter pylori* strains. This infection caused immediate suppression of gastric secretion and delayed the healing of ulcers.

Protell et al. (1976) described a reproducible model of acute bleeding ulcer in **dogs** – the “ulcer maker.” An instrument has been developed for endoscopy or laparotomy, which creates gastric ulcers of reproducible diameter and depth.

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- for ethanol-, indomethacin- and hemorrhagic shock-induced gastric ischemia-reperfusion injuries (Masuda et al. 1993; Kitajima et al. 1993; Michida et al. 1994; Kitajima et al. 1995).

PROCEDURE

Male Wistar rats weighing 200–250 g are fasted for 24 h with free access to water. The rats are anesthetized with 1.5 g/kg urethane i.p. The stomach is exposed by a medial laparotomy and instilled with 0.15 M HCl (1 ml/100 g) via the forestomach. The left gastric artery is clamped by a small vascular clamp for 5 min to induce ischemia and 30 min of reperfusion is done by releasing the clamp. Pretreatment with test drug or standard is given to groups of 5 rats immediately before the induction of ischemia. At the end of the experiment, the rats are sacrificed by cervical dislocation. The stomach is fixed with 10% buffered formalin and photographed for macroscopic evaluation of injuries. For the assessment of microscopic injuries, a sample of corpus 0.5 cm below the limiting ridge containing the entire width of the anterior wall is taken from each stomach and processed for subsequent histological evaluation.

A planimeter attached to a computer is used to trace the macroscopic mucosal injury from color photographs. The results are expressed as a percentage of the total glandular mucosal area.

Each histological section is stained with hematoxylin/eosin and examined under light microscope. An one cm length of each histological section is assessed for epithelial damage (score = 1), glandular disruption, vasocongestion or edema in the upper mucosa (score = 2), hemorrhagic damage in the mid to lower mucosa (score = 3) and deep necrosis and ulceration (score = 4). Each section is evaluated on a cumulative basis to give the histological index, the maximum score thus being 10.

EVALUATION

Data are expressed as mean \pm SEM. Comparisons between different groups are made by one way analysis of variance followed by Fisher's least significant difference test. P-values of <0.05 are considered as statistically significant.

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J.3.7.5

Gastric Ischemia-Reperfusion Injury in Rats

PURPOSE AND RATIONALE

Hassan et al. (1997) described the effect of an endothelin converting enzyme inhibitor on local gastric ischemia-reperfusion injury in rats. Endothelin-1 has potent ulcerogenic effects in the stomach (Wallace et al. 1988). Endogenous endothelin-1 has been implicated

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J.4 Intestinal Functions

J.4.1 Intestinal Secretion

J.4.1.1 Laxative Activity in Rats

PURPOSE AND RATIONALE

Laxatives of the sennoside type act mainly by acceleration of large intestine transit and inhibition of fluid absorption in the colon (Leng-Peschlow 1986).

PROCEDURE

For **measurement of large intestinal transit time**, female Wistar rats weighing approximately 200 g are anesthetized with ether. A PVC catheter is implanted into the caecum with the distal end fixed on the animal's neck. The animals are allowed to recover and are placed individually in a wire meshed cage to enable the feces to fall through onto blotting paper. Carmine red (10 mg in 0.4 ml distilled water per animal) is injected through the catheter immediately after administration of the test substance. The time until appearance of the first colored feces is registered.

For **measurement of fluid absorption in the colon**, female Wistar rats weighing approximately 200 g are anesthetized with 50 mg/kg pentobarbitone sodium. The colon is ligated and cannulated distal to the caecocolic junction (PE-tube, i.d. 1 mm) and, after a thorough rinse with 50 ml physiological saline to remove all contents, a second cannula (silicone, i.d. 3 mm) is inserted proximal to the rectum for fluid outflow. Four and 6 h after oral administration of the test compounds an open perfusion with an electrolyte solution (NaCl 6.72 g/l, KCl 0.37 g/l, NaHCO₃ 2.1 g/l, polyethylene glycol (PEG, mol wt 4000) 2.0 g/l, [¹⁴C]PEG 5 μCi/l; pH 6.5, osmolality 275 milliosmol/kg) is started at a rate of 12 ml/h for two consecutive 2 h periods. [¹⁴C]PEG activity is measured by liquid scintillation counting, Na⁺ and K⁺ by flame photometry, Cl⁻ by

coulometric titration, osmolality by freezing point depression and mucus as protein-bound total hexoses by the orcinol-sulphuric acid method. Net H₂O, Na⁺, K⁺ and Cl⁻ transport are calculated and expressed as ml or μmol/h and per 10 cm colon length.

EVALUATION

All values are expressed as mean ± standard deviation. Statistical significance is assessed with Student's *t*-test.

MODIFICATIONS OF THE METHOD

Ogunti and Elujoba (1993) tested the laxative activity of *Cassia alata* in rats. Male Charles River rats were kept in individual cages during one week. Any rat producing wet feces was rejected. After administration of the test compounds to groups of 5 rats per dose, the feces were examined for wetness hourly for 12 h. The results were expressed as the mean percent of total feces that were wet per kg rat.

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J.4.1.2 Enteropooling Test

PURPOSE AND RATIONALE

The enteropooling assay in rats has been developed by Robert et al. (1976) to test the diarrheogenic property of prostaglandins for prediction of this clinically relevant side effect of several synthetic prostaglandins.

PROCEDURE

Female Sprague Dawley rats weighing 190–215 g are used. The animals are fasted overnight having free access to water. The test compounds are administered orally, and the animals, 12 per group, are sacrificed one hour later. The fluid accumulation occurs in the small intestine which is cut at the pylorus and the ileocecal junction, and its contents, consisting of a thick fluid (in controls) and a very watery fluid (in prostaglandin-treated animals) are collected into a graduated test tube by milking the whole length of the small intestine with the fingers. The volume of fluid is recorded.

EVALUATION

Using various doses, dose-response curves can be established and potency ratios calculated. 16,16-dimethyl PGE₂ was found to be the most active compound.

CRITICAL ASSESSMENT OF THE METHOD

Some other diarrheogenic agents, like MgSO₄, castor oil, bile, taurocholate and taurochenodesoxycholate cause enteropooling, whereas mineral oil and tragacanth are ineffective. The anticholinergic agent methylscopolamine partially counteracted the enteropooling. The assay, therefore, can be used to test the laxative or the antidiarrheal activity of compounds (Shook et al. 1989).

MODIFICATIONS OF THE METHOD

Beubler and Badhri (1990) used the PGE₂-induced net fluid secretion in the jejunum and colon in the rat to evaluate the antisecretory effects of antidiarrheal drugs. Polyethylene catheters were placed into the jejunum and colon and Tyrode solution was instilled into the loops. Net fluid transfer rates were determined gravimetrically 30 min after instillation of Tyrode solution.

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J.4.1.3**Inhibition of Chloride Secretion in Rabbit Colon²****PURPOSE AND RATIONALE**

Epithelia have the ability to reabsorb or to secrete fluids and electrolytes. In 1958, Koefoed-Johnsen and Ussing first published a model of ion transport across frog skin epithelium. Ussing also introduced the measurement of the short-circuit current as a means of defining active ion transport (Koefoed-Johnsen and Ussing 1958). Mammalian colon is an example of epithelium which has the capacity to absorb and to secrete electrolytes.

Previous studies have shown that mammalian colon actively secretes chloride when exposed to prostaglandins and vasoactive intestinal peptides, which increase the cellular concentration of cAMP resulting in an electrogenic chloride secretion (Frizzell et al. 1976). The elevation of cAMP causes the opening of chloride channels, whereas the colonic epithelium absorbs sodium and chloride ions by an electroneutral mechanism under control conditions. In general it is assumed that absorption takes place mainly in the surface cells, whereas the crypts are the predominant site of secretion (Greger et al. 1985; Binder and Sande 1987).

PROCEDURE

Rabbits of either sex (2.0 to 4.0 kg body weight) are killed by cervical dislocation. The distal colon is removed, immediately opened into a flat sheet and washed in the standard electrolyte solution. The epithelium with an area of 1.0 cm² is stripped from its underlying musculature and mounted in an Ussing chamber. The tissue is mounted vertically and bathed on both sides by electrolyte solutions, which are circulated and oxygenated by a water-jacketed bubble-lift apparatus maintained at 37°C. The carbogen gas used for bubbling contains O₂ and CO₂ in a mixture of 95% and 5%, respectively. The solutions used on the two sides have the following composition (in mmol/l): NaCl 120, NaHCO₃ 21, Na₂HPO₄ 0.4, K₂HPO₄ 1.6, MgCl₂ 1.2, glucose 5.

Tissues are continuously short-circuited by a four-electrode automatic voltage-clamp apparatus (AC-Microclamp, Aachen, Germany) which measures short-circuit current (I_{sc}) and automatically subtracts chamber fluid resistance. Transepithelial electrical potential difference is measured between Ag-AgCl electrodes, which make contact with the bathing solutions via agar bridges (5% agar in glucose-free standard electrolyte solution). I_{sc} is measured by passing sufficient current through Ag-AgCl electrodes to reduce the spontaneous transepithelial electrical potential difference to zero. Transepithelial conductance (G_t) is determined by passing 100-ms bipolar current pulses through the tissue.

The standard protocol consists of an initial equilibration period of 20 min. The I_{sc} measured in the presence of indomethacin (1 μmol/l, serosal and mucosal solution) corresponds mostly to the rheogenic reabsorption of sodium. In the next step, amiloride is added at 0.1 mmol/l to block sodium reabsorption. Then PGE₂ (1 μmol/l) is added to the serosal solution in the presence of amiloride in order to stimulate

²Contributed in the first edition by M. Hropot

chloride secretion. After a further equilibration period of 20 min putative blockers of chloride secretion are added to the mucosal or serosal solution.

EVALUATION

Each compound is examined at least 3 times and at three different concentrations (usually 1, 10 and 100 $\mu\text{mol/l}$). From the mean values a concentration-response curve is constructed, and from this curve the IC_{50} value is read as the concentration producing 50% inhibition of the stimulated I_{sc} .

Data are presented as means \pm SEM. Paired t -test with a significance level of $p < 0.05$ may be used.

CRITICAL ASSESSMENT OF THE METHOD

The method can be used to study the antidiarrhoic activity of a test compound, but also generally its influence on active electrolyte transport across the cell membrane.

MODIFICATIONS OF THE METHOD

Warhurst et al. (1996) studied the effects of somatostatin analogues on electrogenic ion secretion in isolated rat colonic mucosa mounted in Ussing chambers.

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J.4.2

Antidiarrhea Effect

J.4.2.1

Castor Oil Induced Diarrhea

PURPOSE AND RATIONALE

The induction of diarrhea with castor oil results from the action of ricinoleic acid formed by hydrolysis of the oil (Iwao and Terada 1962; Watson and Gordon 1962). Ricinoleic acid produces changes in the transport of water and electrolytes resulting in a hypersecretory response (Ammon et al. 1974). In addition to hy-

persecretion, ricinoleic acid sensitizes the intramural neurons of the gut.

PROCEDURE

Female Wistar rats weighing 210–230 g are used after overnight food deprivation. For the experiment, the rats are housed in individual cages with no access to drinking water. The potential antidiarrhoic agents are administered orally by gavage in various doses. Controls receive the solvent only. Each dose is given to 10 animals. One hour after dosage, 1 ml of castor oil is administered orally. Stools are collected on non-wetting paper sheets of uniform weight up to 24 h after administration of the castor oil. Every 15 min during the first 8 h, urine is drained off by gravity, and the net stool weight, termed early diarrheal excretion, is recorded. The diarrhea-free period is defined as the time in minutes between castor oil administration and the occurrence of the first diarrheal output. The acute diarrheal phase is the time between the first and the last diarrheal output of the 8-h observation period. Stools occurring between 8 and 24 h after castor oil administration are called late diarrheal excretion.

EVALUATION

With antidiarrheal agents dose-response curves are obtained for decrease of hypersecretion (stool weight) and for increase of the diarrhea-free period are obtained. Inhibitors of prostaglandin biosynthesis increase the diarrhea free period but do not affect early diarrheal secretion (Niemegeers et al. 1984).

MODIFICATIONS OF THE METHOD

Inhibition of castor oil-induced diarrhea in **mice** was tested by Bianchi and Goi (1977).

Dajani et al. (1977) tested antidiarrheal activity in castor-oil treated **monkeys**.

Mannitol-induced diarrhea was used as model in **calves** (Fioramonti and Buéno 1977) and in **pigs** (Théodorou et al. 1991).

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J.4.2.2

Antidiarrheal Effect in Cecectomized Rats

PURPOSE AND RATIONALE

Evaluation of antisecretory antidiarrheal agents in animal models is limited primarily to extrapolations of efficacy from enteropooling studies *in vivo* (DiJoseph et al. 1984), isolated intestinal loops (Nakaki et al. 1982), and Ussing flux chamber preparations *in vitro* (Dharmasathaphorn et al. 1984). These studies do not mimic secretory diarrhea. The method of Magnus (1915) using chronic diarrhea in cats induced by continuous milk diet and thereby proofing the antidiarrheal effect of morphine is of historical interest only.

Fondacaro et al. (1990) developed a model of secretory diarrhea utilizing conscious cecectomized rats by surgical resection of the cecum and by use of potent intestinal secretagogues. The rat has a pronounced cecum as part of its gastrointestinal tract. The rat cecum is not only a reservoir for intestinal contents where high concentrations of various microbial flora assist

in the digestion of carbohydrates, cellulose and peptides through microbial fermentation processes (Ambuhl et al. 1979; Williams and Senior 1982) but also plays a role in handling of excess intestinal fluid.

PROCEDURE

Cecectomies are performed in unfasted rats weighing 200–250 g. Under anesthesia with methohexital (60 mg/kg i.p.) cecectomy is initiated with a 2-cm mid-ventral incision. The cecum is lifted from the abdominal cavity and exteriorized onto a gauze drape. The cecal apex is freed by severing the avascular area of the mesocecum. A ligature of no. 1 silk suture is positioned so as to occlude the cecum and its vasculature without compromising ileo-colonic patency. After the ligature is secured and ileo-colonic patency confirmed, the cecum is resected, and the remaining exposed cecal mucosa is washed with saline and cauterized. The intestinal segment is then returned to the abdominal cavity and the abdominal muscle fascia closed with sutures. The dermal incision is closed with wound clips that are removed about 1 week postsurgery. Immediately following the surgical procedure, the animals are returned to their cages and allowed free access to food and water. The animals are permitted at least a 48-h recovery period before being used in an experiment.

For the diarrhea assay, cecectomized rats are put into individual wire-bottomed cages placed over sheets of clean paper, and deprived of food and water for the duration of the assay. Rats are given a two hour's acclimatization period prior to the start of the assay in order to eliminate sporadic episodes of anxiety-induced defecation. During this period, they are observed also for consistent occurrences of pelleted feces; an animal producing other than pelleted stool is disqualified from the study. Diarrhea is induced with oral administration of secretagogues: either 16,16 dimethyl prostaglandin E₂ (0.3 mg/kg) in 3.5% ethanol, carbachol (15 mg/kg) in water, or cholera toxin (0.5 mg/kg) in an aqueous vehicle of 2% NaHCO₃ plus 2% casamino acids. Anti-diarrheal agents are administered by gavage after the onset of diarrheal episodes. The cage papers are removed and examined at 15 min intervals for carbachol-induced diarrhea, 30 min intervals for 16,16 dimethyl prostaglandin E₂ induced diarrhea, and hourly when cholera toxin is used as secretagogue. Fecal output is recorded at each interval and scored as follows:

- 1 = normal pelleted stool
- 2 = soft-formed stools
- 3 = water stool and/or diarrhea

Known antidiarrheal agents, such as chlorpromazine (10 mg/kg p.o.), or the alpha-2 receptor agonist clonidine (1.0 mg/kg p.o.), or morphine (10 mg/kg p.o.) reduce the fecal output and induce a cessation of diarrhea.

EVALUATION

The fecal output index is defined as the summation of the number of defecation periods and their ranked consistency score within an observation period and is expressed as mean \pm SEM for each group. Student's *t*-test and analysis of variance are used for statistical comparisons of data points. Significance is accepted at $p < 0.05$ or less.

CRITICAL ASSESSMENT OF THE METHOD

The model of diarrhea induced by secretagogues in cecectomized rats has the advantage to mimic secretory diarrhea in man.

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J.4.2.3

Evaluation of Antidiarrheal Effect in Cold-Restrained Rats

PURPOSE AND RATIONALE

Barone et al. (1990) tested the effect of various antidiarrheal and other drugs on increased fecal pellet

output in cold-restrained rats resembling clinical observations that stressful situations can produce diarrhea in humans.

PROCEDURE

Male Sprague-Dawley rats weighing 260–310 g are maintained on Purina lab chow and water. Since gastric ulcers are reduced if cold-restrained rats are allowed free access to food and water, for studies on fecal output food was not withdrawn prior to the experiment. The rats are studied in normal living cages at room temperature (control, non-stressed animals) or in wire-mesh restraining cylinders placed in a cold (4°C) environment. Test drugs are administered by appropriate routes over optimal effective dose-ranges for their activities and at optimal pretreatment times to maximize their effects. The number of pellets expelled by each animal is measured at 1 and 3 h (fecal pellet output). Generally, the fecal pellets of stressed animals are less firm. Fecal pellet fluid content is determined by weighing fecal pellets, drying them in an oven at 37°C, and weighing them again.

EVALUATION

The dose in mg/kg that inhibits the cold restrained stress induced increase in fecal pellet output by 50% (ID_{50}) is determined using least-squares fit analysis directly from the regression line. If fecal pellet output is decreased by a drug but no clear dose-related effects occur, the maximum percent decrease is determined.

MODIFICATIONS OF THE METHOD

For colonic transit studies, rats are implanted with indwelling catheters in the proximal colon. Animals are anesthetized with 60 mg/kg pentobarbital i.p., and a chronic colonic catheter is positioned to enter the proximal colon 2 cm from the ileocecal junction. A catheter of about 20 cm of silicone tubing prepared with several drops of silicon rubber adhesive coating a 1-cm length along the catheter is positioned into the colon. The colon end of the tubing also is sealed with a 1-cm plug of petroleum jelly intraluminally. A small incision is made in the proximal colon, and the adhesive-coated portion of the Silastic tubing is tied in place with the use of a purse-string suture. The tubing is brought through the abdominal wall, led subcutaneously through the skin in the midscapular region and secured on the back of the neck with the use of a wound clip. The abdominal incision is closed with sutures and wound clips.

Experiments are performed in conscious animals 48–72 h after surgical preparation. At this time, the

radiolabeled marker (^{51}Cr as sodium chromate) is instilled into the proximal colon via the indwelling catheter. After 35, 60, or 120 min, cold-restrained rats and controls are sacrificed and their colons and large intestines are removed. The cecum and equal segments of the colon are dissected, placed into vials and subjected to gamma counting.

Ikeda et al. (1995) investigated the effect of a neurokinin₁ receptor antagonist on stress-induced defecation in rats placed in special restraint cages.

Kishibayashi et al. (1993) studied distal colonic function using wrap-restrained stress-induced defecation as described by Williams et al. (1988). The rats were lightly anesthetized with ether, and the fore-shoulders, upper forelimbs and the thoracic trunk were wrapped in paper tape to restrict, but not prevent, movement. The animals recovered from anesthesia within 2–5 min and immediately moved around in cages and ate and drank, but had been restricted from mobility of forelimbs, which prevented them from grooming the face, upper head and the neck. Fecal pellet output induced by wrap-restraint stress was weighed during the 1st h after stress. The test drugs were given p.o. 1 h before stress. The ID_{50} values were calculated as the doses that reduced stress-induced defecation by 50%.

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J.4.3

Gut Motility

J.4.3.1

Isolated Ileum (MAGNUS Technique)

PURPOSE AND RATIONALE

The isolated ileum, as first described by Magnus (1904), is probably the most widely used model in experimental pharmacology. Magnus already studied simultaneously the spontaneous contractions of the longitudinal and circular musculature and the inhibit-

ing effect of atropine. The method has been used for many purposes, such as the study on the effects of adrenaline on the lower segments causing contraction and on the segments of the upper end causing relaxation by Munro (1951) or the study on the origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips by Paton and Zar (1968) either retaining or being denervated from Auerbach's plexus. The model is used as a basic screening procedure for spasmolytic activity, whereby an anti-acetylcholine or anticholinergic effect indicates antimuscarinic activity and an anti-BaCl₂-effect indicates a musculotropic, papaverine-like effect. In addition to the isolated ileum, other parts of the gut such as the isolated duodenum and colon, have been used widely.

PROCEDURE

Guinea pigs of either sex weighing 300 to 500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, with the mesentery being cut away as necessary. When the colon is reached, the intestine is cut. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode's solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 2–3 cm length are cut. Preferable the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into a 15 ml organ bath containing Tyrode's solution at 37°C being oxygenated with 95% O₂/5% CO₂. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA). A preload of 1 g is chosen. Responses are recorded on a polygraph. After a pre-incubation time of 30 min, the experiment is started.

The following agonists and antagonists (standards) are used (concentrations in g/ml bath fluid):

| Agonist | Antagonist |
|--------------------------------------------|---------------------------------------------------------------------------------------------------------|
| Acetylcholine 10 ⁻⁷ g/ml | Atropine 10 ⁻⁸ –10 ⁻⁹ g/ml Scopolamine 10 ⁻⁸ –10 ⁻⁹ g/ml |
| Carbachol 10 ⁻⁷ g/ml | Atropine 10 ⁻⁸ –10 ⁻⁹ g/ml |
| Histamine 10 ⁻⁶ g/ml | Histamine antagonists |
| BaCl ₂ 10 ⁻⁴ g/ml | Papaverine 10 ⁻⁵ –10 ⁻⁶ g/ml |
| Serotonin 10 ⁻⁶ g/ml | Serotonin antagonists |
| PGE ₂ 2 × 10 ⁻⁷ g/ml | PG-antagonists |

EVALUATION

Several methods for the quantitative evaluation of an antagonistic effect are available. One approach is the determination of pD'_2 values according to van Rossum and van den Brink (1963). Acetylcholine or histamine is added in $1/2 \log_{10}$ concentration increments until a maximum response is obtained. Control curves are recorded at 30 min intervals. After uniform control responses are obtained, the potential antagonist or the standard is added 5 min before the concentration-response curve is re-obtained. The potency of the antagonist is obtained by calculating the pD'_2 value which is defined as the negative logarithm of the molar concentration of an antagonist that causes a 50% reduction or the maximal response obtained with an agonist.

MODIFICATIONS OF THE METHOD

Many modifications of the Magnus technique have been described in the literature, mainly with the isolated ileum (e. g., Koelle et al. 1950).

Okwuasaba and Cook (1980) dissected the myenteric plexus and longitudinal muscle free of the underlying circular muscle according to the method of Paton (1957), Paton and Zar (1968) and stimulated the preparation with trains of supramaximal rectangular pulses of 1.0 ms duration at a frequency of 0.2 Hz.

Kilbinger et al. (1995) studied the influence of 5-HT₄ receptors on [³H]-acetylcholine release from guinea pig myenteric plexus.

De Graaf et al. (1983) described a fully automated system for *in vitro* experiments with isolated tissues. The apparatus consists of an organ bath equipped with (a) a gradient pump supplying a logarithmic concentration/time gradient of agonist; (b) pumps and valves for dispensing bath fluid, antagonist solutions, and an oxygenation gas mixture; and (c) a transducer with automatic baseline adjustment. The information coming from the preparation is fed into a mini-computer. The data of various experiments can be accumulated and Schild-plots obtained.

Furukuwa et al. (1980) studied the effects of thyrotropin-releasing hormone on the isolated small intestine and taenia coli of the guinea pig.

Paiva et al. (1988) studied the role of sodium ions in angiotensin tachyphylaxis in the guinea-pig ileum and taenia coli.

Barnette et al. (1990) used electrically stimulated strips of circular smooth muscle from the lower esophageal sphincter of dogs to study the inhibition of neuronally induced relaxation by opioid peptides.

Bradykinin antagonism can be studied in the isolated guinea pig ileum bathed in a solution contain-

ing atropine (1.5 mM), diphenhydramine (3.4 mM), indomethacin (2.8 mM), and captopril (0.9 mM) (Rubin et al. 1978; Kachur et al. 1987).

Griesbacher and Lembeck (1992) used the isolated guinea-pig ileum for analysis of bradykinin antagonists.

Hew et al. (1990) used field stimulated (95% of maximum voltage, 0.1 Hz, 0.5 ms) guinea pig ileum, bathed in physiological salt solution at 37°C in the presence of 1 mM mepyramine for determination of histamine H₃ bioresponse. Reduction of contractile response by the test substance (>50% relative to control 0.3 mM R- α -methylhistamine) indicates possible H₃ agonism. At a test concentration where no significant activity is seen, ability to inhibit (>50%) R- α -methylhistamine-induced contractile reduction indicates antagonistic activity.

A similar technique was used by Conner et al. (1987) to study antagonist effects at functional 5-HT_{1A} receptors.

Feniuk et al. (1993) used the guinea-pig isolated ileum, vas deferens and right atrium to characterize somatostatin receptors. Transmural electrical stimulation was applied to guinea pig ileum (0.1 Hz, 0.1 ms continuously) and vas deferens (5 Hz, 0.5 ms for 1.5 s every 30 s) at supramaximal currents (approximately 800 mA) delivered from a Digitimer D330 multistimulator.

Radomirow et al. (1994) investigated opioid effects of short enkephalin fragments containing the Gly-Phe sequence on contractile responses of guinea pig ileum after addition of 10 nM acetylcholine or after electrical stimulation.

Coupar and Liu (1996) described a simple method for measuring the effects of drugs on intestinal longitudinal and circular muscle in **rats**. The preparation consists of a segment of rat ileum set up to measure the tension developed in the longitudinal muscle and intraluminal pressure developed in the circular muscle in response to transmural electrical stimulation.

Vassilev et al. (1993) exposed Wistar rats to subtoxic doses of Co²⁺ or Ni²⁺, receiving Co(NO₃)₂ or NiSO₄ with drinking water for 30 days, and measured the changes in the contractile responses to carbachol and in the inhibitory effects of verapamil and nitrendipine on isolated smooth muscle preparations of the ileum and the trachea.

Pencheva and Radomirov (1993), Pencheva et al. (1999) studied the effects of GABA receptor agonists on the spontaneous activity of the circular layer in the terminal ileum of **cats**. Segments of the terminal ileum approximately 0.5 cm long were mounted in an organ

bath along the axis of the circular layer through a cotton thread with a large knot situated at the inner part of the gut wall.

Similar preparations of cat ileum were used by Kortežova et al. (1994) and Chernaeva and Mizhorkova (1995).

Vassilev and Radomirov (1992) used an isolated preparation of **rat rectum**. The rectal region, 1–6 cm proximal to the anal sphincter was removed and a 20 mm long segment suspended in an organ bath. The influence of prostaglandins and antagonists on spontaneous mechanical activity and electrically stimulated responses was investigated.

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J.4.3.2

Cascade Superfusion Technique

PURPOSE AND RATIONALE

The technique of isolated organ superfusion was developed by Gaddum (1953) for the assay of biologically active substances. Extension of the technique for multiple tissue superfusion with particular reference to the identification and the assay of prostaglandin-like activity was used by various authors (Vane 1964;

Ferreira and Vane 1967; Ferreira et al. 1976; Gilmore et al. 1968; Hong 1974; Bult et al. 1977; Henman et al. 1978; Elliott and Adolfs 1984; Fournau et al. 1984).

PROCEDURE

The apparatus consists of a double-wall glass container (height 20–25 cm, inner diameter 7–8 cm) with an outlet at the bottom. A constant temperature of 38°C is maintained by circulation of warm water through the outer jacket. Inside the glass container can be suspended up to 5 pieces of tissue of various origins. The multiple preparation tissue holder consists of a vertical rectangular rod and plastic platforms for attachment of the tissues and for accurate deflection of the superfusate on to the lower tissue. The rod is grooved at 10 mm intervals with 1 mm deep slots set at an angle of 20° to the horizontal. To the upper surface of the nonwetable platform a small plastic hook is cemented at such a distance from the rod that when the tissue is in position its attachment thread passes between the V-shaped notch cut into the margin of the upper tissue platform. The individual platforms are inserted on to the vertical rod by slotting into the requisite grooves appropriate to the tissue length.

Thus, the superfusate passes at a uniform flow rate down the tissues of the cascade and the tension recording threads are separated from each other by about a 5-mm gap so that the responses can be conveniently recorded. The threads from the organs are connected over isotonic levers to isometric tension transducers. The lever is used for preloads according to the individual organ. Tension exerted by each tissue is recorded on a polygraph. Various media can be used for superfusion, e. g., Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂.

Many tissue preparations can be used for the cascade, such as rat fundic strip, rat duodenum, rat colon, rat bladder strip, guinea-pig ileum, guinea-pig proximal colon, rabbit stomach strip, rabbit coeliac/mesenteric artery, or rabbit aorta strip. Moreover, donor tissue can be superfused and its effluent be tested in the organs of the cascade.

EVALUATION

Many agonists and antagonists can be tested by appropriate selection of organs. Threshold doses and *ED*₅₀ or *ID*₅₀ values can be determined.

MODIFICATIONS OF THE METHOD

The superfusion technique has been used for several purposes, e. g., for the assay of catecholamines (Armitage and Vane 1964), for detecting active substances

in the circulating blood (Vane 1964). A simple and inexpensive piece of apparatus for cascade superfusion procedures has been described by Naylor (1977).

Mombouli et al. (1996) described a bioassay of endothelium-derived hyperpolarizing factor (EDHF) using a perfusion-superfusion cascade where canine carotid arteries were used as donors of vasoactive substances and rings of coronary arteries without endothelium as detectors.

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J.4.3.3

In Vivo Evaluation of Spasmolytic Activity in Rats

PURPOSE AND RATIONALE

Maggi and Meli (1982) described an *in vivo* procedure for estimating spasmolytic activity in the rat by measuring smooth muscle contractions to topically applied acetylcholine.

PROCEDURE

Male albino rats weighing 350–400 g are anesthetized with subcutaneous urethane (1.2 g/kg). The left jugular vein is cannulated for administration of test compounds. After laparotomy occluding silk ligatures are applied at a distance of 2 cm from each other in the gut (colon or rectum). Through a small incision the flanged tip of a polyethylene tubing (1 mm i.d., 1.5 mm o.d.) is inserted into the lumen of this pocket-like space and secured in place by a purse string ligature. The free end of the tubing is connected to a pressure transducer and the whole system filled with saline. The same procedure is performed with the urinary bladder. The organs are filled with warm saline (37°C) to obtain a resting pressure of 4–12 mm Hg. Warm saline soaked cotton wool swabs are laid around the exteriorized organs which are maintained warm and moist with warm (37°C) saline dropping from a reservoir at a rate of 10–15 drops/min.

After 15 min stabilization period, saline flow is stopped and a dose-response curve to acetylcholine determined. A volume of 0.5 ml (an amount sufficient to put the whole outer surface of the organ into contact with the bathing solution) of acetylcholine at the desired concentration is applied within 2–3 s from a syringe to the outer surface of the target organ. To construct a dose-response curve of acetylcholine, increasing concentrations are applied to the target organ until maximal contraction is obtained. After at least 3 or more control curves have been obtained at 10 min intervals, the antagonist is administered.

EVALUATION

The quantitative analysis of the data is carried out by plotting the results of each experiment as log (acetylcholine dose ratio-1) against log dose antagonist (Arunlakshana and Schild 1959). The regression line is calculated according to the method of least squares and ED_{50} values and 95% confidence limits according to Litchfield and Wilcoxon (1949). From ED_{50} values, the dose of the antagonist (mg/kg i.v.) to produce an acetylcholine dose ratio of 10 is calculated according to Daly et al. (1975). Parallel displacement to the right of the agonist dose response curve and a slope of unity for the regression line indicate competitive antagonism. The DR_{10} values from different organs are compared by means of Student's *t*-test for unpaired data.

MODIFICATIONS OF THE METHOD

In vivo registration of gut motility in guinea pigs was already described by Straub and Viaud (1933). A four

cm long part of the gut was ligated and filled with Tyrode solution. Gut motility was measured at variable intraluminal pressure.

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J.4.3.4**Colon Motility in Anesthetized Rats****PURPOSE AND RATIONALE**

The influence of spasmolytic drugs on carbachol induced increase of colonic motility can be measured in anesthetized rats. The method has also been used to study the stimulation of colonic motility by an enkephalin analogue pentapeptide (Bickel 1983).

PROCEDURE

Male Sprague-Dawley rats weighing 350–500 g are anesthetized with pentobarbital i.v. A pressure sensitive tip catheter is inserted into the colon ascendens and the signals of the intraluminal pressure changes are recorded. The colonic contractions are stimulated by i.v. injection of 3 mg/kg carbachol. The height and the duration of the contractions are recorded. Then, the test compound is injected intravenously. The decrease of contractions is measured and the duration of the spasmolytic activity determined by repeated administration of carbachol at 15 min intervals, until the contractions are not significantly different from the response obtained with carbachol alone.

EVALUATION

Significant differences are calculated using Student's unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Maggi and Meli (1984) used eserine-induced hyper-tonus of **guinea pig** distal colon *in vivo* as a phar-

macological procedure for testing smooth muscle relaxants. Male albino guinea pigs weighing 240–300 g are anesthetized with 1.5 g/kg urethane s.c. Through a midline abdominal incision, the proximal part of the hypogastric loop of the distal colon is exposed and occluding silk ligatures are applied at a distance of 2 cm from each other, taking great care to avoid any lesion to the vascular and nervous supply. Through a small incision, the flanged tip of a polyethylene tube (1 mm i.d., 1.5 mm o.d.) is inserted into the lumen and secured by means of a purse-string ligature. The free end of the tube is connected to a pressure transducer and the whole system is filled with saline. Intraluminal pressure and its variations are recorded on a polygraph. The effect of drugs is assessed as inhibition of eserine induced hypertonus.

Théodorou et al. (1991) studied the absorptive and motor components of the antidiarrheal action of loperamide in **pigs**. Motility was recorded by implantation of intraparietal electrodes into various parts of the gut.

Raffa et al. (1987) used a method utilizing the insertion of a 3 mm glass bead into the distal colon in **mice** to evaluate the activity of intracerebroventricularly administered μ - and δ -opioid agonists on colonic bead expulsion time.

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J.4.3.5

Continuous Recording of Electrical and Mechanical Activity in the Gut of the Conscious Rat

PURPOSE AND RATIONALE

Bueno et al. (1981) described a method for continuous electrical and mechanical activity recording in the gut of the conscious rat. In this study two methods for the continuous recording of motor events – microtransduc-

ers and the electromyogram – are compared for the rat stomach and intestine.

PROCEDURE

Male Wistar rats weighing 200–300 g are housed singly in wire-bottomed cages and allowed lab chow and water ad libitum during the training period of 30 days. Under halothane anesthesia pairs of electrodes are implanted along the greater curvature of the pyloric antrum 5 and 2 cm from the pylorus, on the antimesenteric border of the duodenum 2 and 5 cm beyond the pylorus, and along the jejunum, 4 cm from the pylorus using a procedure described by Ruckebusch and Fioramonti (1975).

The contractions of the circular muscle layer are recorded with strain gauge microtransducers sutured onto the serosa at less than 1 cm from each set of electrodes (Pascaud et al. 1978). The free ends of the electrodes and strain gauge wires are carried subcutaneously and exteriorized on the back of the neck. The wires are inserted into a glass tube (15 cm and 0.5 cm external diameter) to prevent twisting and any contact with the metallic lids of the cage.

EVALUATION

Both electrical and mechanical activities are continuously recorded starting 5–7 days after surgery on a multichannel recorder (e.g., 8-channel Dynograph, Beckman, USA) using lead selector couplers (type 9856, Beckman) at a constant time of 0.1 s for the electrical spiking activity and strain gauge couplers (type 9863, Beckman) for the contractile force minitransducers. Simultaneously low frequency signals (frequency < 3 Hz) of the electromyogram are eliminated through filters and the spiking activity, integrated for each 20 s, is recorded on a potentiometric recorder. The electrical and mechanical activities are also recorded simultaneously on a magnetic tape recorder.

The index of motility, expressed as mcoul/min is calculated from the integrated records of electrical activity. The index of mechanical motility is calculated as the area under all contraction waves occurring during 1 min, and is expressed as gs/min. Mechanical and electrical activities during a test period of 30 min after administration of stimulant drugs, e.g., the gastrointestinal hormones gastrin and cholecystokinin, or relaxing drugs, like anticholinergic agents, are compared with the values of a 30 min pretest period.

MODIFICATIONS OF THE METHOD

Wright et al. (1981) described a similar method for long-term recording of intestinal mechanical and elec-

trical activity in the unrestrained rat. Mechanical activity is detected using miniaturized half-bridge metal foil strain-gauge force transducers. The electrical activity is monitored by silver/silver chloride bipolar electrodes. The lead wires from the recording units are encased in a metal compression spring and are permanently joint to a ball connector positioned on the top of the cage, such allowing the animal free access to all parts of the cage.

Stam et al. (1995) described computer analysis of the migrating motility complex of the small intestine recorded in freely moving rats. Myoelectric activity of the small intestine was recorded digitally in fasted, freely moving rats with multiple pairs of electrodes in the antimesenteric smooth muscle. A computer program was developed to distinguish the three characteristic phases of the migrating motility complex.

Fändriks (1993) measured duodenal wall motility, mucosal fluid transport and alkaline secretion in anesthetized **cats**. A triple-lumen tube supplied with two small balloons was positioned via the esophagus in the duodenal lumen and its distal end was led through a small incision in the antimesenteric border of the distal duodenum at the level of the ligament of Treitz. The most oral of the balloons was positioned immediately to the pylorus. After filling with air, the balloons occluded the lumen and isolated a 2 cm segment of the proximal duodenum. A double-lumen tube was inserted into the stomach for luminal perfusion.

Martinez et al. (1993), Jimenez et al. (1994) studied gastrointestinal motility and coordination in **chickens**. Animals were chronically implanted with electrodes in stomach, duodenum, ileum, caeca and rectum.

Nakajima et al. (1996) used a telemetric device (supplied by Data Sciences International, Inc., St Paul, MN) which can be implanted in the abdominal cavity of small animals. Gastric motility of freely moving rats could be continuously recorded for up to 60 days.

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J.4.3.6

Propulsive Gut Motility in Mice

PURPOSE AND RATIONALE

The passage of a charcoal meal through the gastrointestinal tract in mice is used as parameter for intestinal motility and to study the effect of laxatives.

PROCEDURE

Groups of 10 female mice (e. g., NMRI strain) weighing 15 g are fed an oat diet for 3 days. Eighteen hours prior to the experiment food, but not water, is withdrawn. The animals are treated either subcutaneously 15 min or orally 60 min before administration of the charcoal meal (0.2 ml of a 4% suspension of charcoal in 2% carboxymethylcellulose solution). The mice are sacrificed after various time intervals, 20 min, 40 min, 60 min and 120 min. Ten animals serve as controls for each time interval. The entire intestine is immediately removed and immersed in 5% formalin to halt peristalsis; then washed in running water. The distance the meal has traveled through the intestine as indicated by the charcoal is measured and expressed as percent of the total distance from the pylorus to the caecum.

EVALUATION

Student's *t*-test is used to compare the control and the drug-treated group.

CRITICAL ASSESSMENT OF THE METHOD

The charcoal passage test can be used for evaluation of laxative activity as well as for inhibition of intestinal motility.

MODIFICATIONS OF THE METHOD

Instead of charcoal, unsubstituted Hostapermblau (CuPcB) suspended in gummi arabicum mucilage can be used.

Carmine red (15) suspended in a 1% tragacanth solution was used for measurement of small intestine transit in **rats** (Leng-Peschlow 1986).

Miller et al. (1981) measured the intestinal transit in the rat by the use of radiochromium (^{51}Cr). Female Sprague Dawley rats weighing approximately 200 g were implanted with indwelling silastic cannulae in the proximal duodenum. Following a 3 day recovery period, the animals were fasted for 18 h and then treated with the test compounds. Thirty min later, 0.2 ml of radiochromium (0.5 mCi $\text{Na}^{51}\text{CrO}_4$) was instilled into the small intestine via the indwelling silastic cannula. Twenty-five min after chromium instillation, the animals were sacrificed. The small intestine was carefully removed and divided into 10 equal segments. The radioactivity was determined with an automatic gamma counting system. The effect of drugs could be quantified by determining the geometric center of the distribution of chromium through the small intestine.

Shook et al. (1989) used radiolabeled chromium to measure gastrointestinal transit in mice.

Megens et al. (1989) used the charcoal test to study the *in vivo* dissociation between the antipropulsive and antidiarrheal properties of opioids in rats.

Lish and Peters (1957) recommended an intestinal antipropulsive test in intact insulin-treated rats providing certain advantages over the commonly used charcoal meal test for screening of synthetic antispasmodic and antipropulsive agents.

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J.4.3.7

Nerve-Jejunum Preparation of the Rabbit

PURPOSE AND RATIONALE

Perivascular nerve stimulation induces cessation of peristalsis of rabbit jejunum (Finkelman 1930). Effects of test compounds on this phenomenon can be tested.

PROCEDURE

Albino rabbits are sacrificed and the jejunal part of the gut prepared. The nerve lies in the mesentery along with the arterial blood supply. Nerve-jejunum preparations are suspended in an organ bath. The preparation is stimulated with pulses at a frequency of 20 Hz with 0.5 ms and about 5 volt for 10 s at 3 min intervals. Test compounds are applied to the organ bath in a cumulative manner at 6 min intervals. Peristalsis movements for each period of 3 min between drug application or of the period of cessation of peristalsis induced by nerve stimulation of the rabbit jejunum preparation are recorded. The effect of drugs on spontaneous peristalsis movement and on the cessation of peristalsis movement exerted by perivascular nerve stimulation is tested.

EVALUATION

The areas of peristalsis movement for each period of 3 min are measured using a planimeter. The effects are expressed as percentage of change between controls and test compound treated preparations.

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J.4.3.8

Motility of Gastrointestinal Tract in Dogs

PURPOSE AND RATIONALE

Intraluminal pressure and motility of the small intestine can be measured in unanesthetized dogs with balloon catheter systems via a duodenal Mann-Bollman (1931) fistula according to Tasaka and Farrar (1976) or in the loop of a Thiry–Vella fistula (see below).

PROCEDURE

Fistulas of the small intestine are created in male Beagle dogs weighing 15–20 kg. The animals are anesthetized with 40 mg/kg pentobarbital i.v. and fixed on an operation table. After shaving and careful disinfection of the skin a midline incision is made. A 10–15 cm length of ileum, approximately 15 cm proximal to the caecum, is excised. The remaining ileum is anastomosed end-to-end. The excised ileum is anastomosed, end to side, to the proximal or middle jejunum. Radiopaque tantalum markers are sutured to the serosa distal to this anastomosis in order to guide the direction for subsequent intubation. The other end is sutured to the skin. To create a skin ileostomy which does not shrink rapidly, a small amount of muscle, fascia and subcutaneous tissue are excised from the abdominal wall.

For the measurement of the pressure inside the intestine, an air-filled system is employed. Air-filled latex balloons (Cementex), 5 mm in diameter, are attached to air-filled PE 190 polyethylene catheters (ID 1.19 mm) with a length of 120 cm. Three balloon-catheter pressure assemblies are tied together with the balloons 5 cm apart. The catheters are connected to Statham p23 db pressure transducers and to a polygraph. The transducers are rendered airtight by repeated applications of latex to all the connections.

The dogs are withheld from food, but not from water 18 h prior to the experiment. The balloon-catheter assemblies are introduced through the fistula and secured in an appropriate position. The system is filled with air to a pressure of 10 mm Hg. Similarly, balloon-catheter assemblies can be introduced into a Thiry–Vella fistula.

Intraluminal pressure is measured continuously; frequency and amplitude of pressure waves are recorded. After a period of 1 h, the test drug is administered orally or subcutaneously and the above mentioned parameters recorded for 10 min intervals.

EVALUATION

For 10 min intervals amplitude frequency (f_A), average degree and duration of amplitudes (A) and average pressure performance (PL_i) are calculated. Post-drug values are compared with pre-drug readings.

MODIFICATIONS OF THE METHOD

Goldenberg and Burns (1973) reported on a technique using rubber balloon catheters inserted in the duodenum, ileum, or colon of dogs, secured by purse-string sutures and filled with water to record intraluminal pressure monitored by Statham pressure transducers

connected to a polygraph. Furthermore, an antispasmodic agent can be tested for relaxation of morphine sulfate (0.3 mg/kg i.v.) induced spasms of the intestinal tract.

Fox et al. (1985) implanted chronically extraluminal strain gage force transducers on the serosal surface of the gastrointestinal tract of dogs.

REFERENCES AND FURTHER READING

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J.4.3.9**Thiry–Vella Fistula****PURPOSE AND RATIONALE**

As described first by Thiry (1864) and improved by Vella (1892), a part of the jejunum is isolated and the ends are exteriorized through the abdominal wall allowing to measure *in vivo* motility and function of the intestines in dogs and other species (Boldyreff 1925).

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. They are fasted 24 h preoperatively. Intravenous pentobarbital sodium, 30 mg/kg, provides satisfactory anesthesia. It can be supplemented, if necessary, during operation. The abdominal part is shaved with electric clippers, then with a razor. The skin is disinfected with Zephiran–70% alcohol. Sterile drapes are applied to cover the whole operative field. A mid-line linea alba incision is made. A loop of the jejunum, about 70 cm in length, is separated leaving the blood supply through the mesenterium intact. Both distal and proximal ends are exteriorized through the abdominal wall and provided with stomata. An end-to-end jejuno-jejunal anastomosis is performed.

EVALUATION

The preparation can be used to evaluate intestinal motility. A latex balloon connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB) is introduced through the proximal ostium. Changes in

intra-gastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of intestinal motor activity. Secretin inhibits motility of the small intestine dose-dependent.

MODIFICATIONS OF THE METHOD

Sarr et al. (1981) and Bastidas et al. (1990) used **dogs** with Thiry–Vella loops to study jejunal absorption.

Bilchik et al. (1993) examined the effects of physiological doses of peptide YY in dogs with jejunal and ileal exteriorized, neurovascularly intact Thiry–Vella fistulas.

Ashton et al. (1996) created Thiry–Vella fistulas using a 20 cm segment of distal colon under general anesthesia in dogs. Colonic absorption of water and electrolytes was evaluated in awake, conscious animals.

Liu et al. (1996a) studied the effects of intravenous peptide YY on colonic water and electrolyte transport in awake dogs which had 20 cm neurovascularly intact colon Thiry–Vella fistulas.

In an other study, Liu et al. (1996b) evaluated the effects of cholecystokinin and peptide YY on intestinal absorption of water and electrolytes using colonic, ileal, or jejunal fistulas in dogs.

These authors (Liu et al. 1995) also examined the effects of intraluminal administration of a new substituted peptide YY analog on intestinal absorption of water and electrolytes in dog with jejunal, ileal and colonic Thiry–Vella fistulas.

Barry et al. (1995) investigated the effect of luminally administered dopamine, the D₁-receptor agonist SKF 38393, the α_1 -receptor antagonist terazosin, and the α_2 -receptor antagonist yohimbine on ileal water and electrolyte transport in dogs with Thiry–Vella fistulas.

Walters et al. (1994) described the effect of a model of canine jejuno-ileal orthotopic autotransplantation on jejunal and ileal transport of water and electrolytes. For neurally intact jejunal loops, myoneural continuity between the loop and the proximal duodenum and jejunum was maintained by preserving a bridge of tunica muscularis devoid of mucosa between the proximal jejunum and the loop. All intrinsic neural and lymphatic continuity to the loop was carefully maintained by not transecting any of the mesentery to the loop.

For the autotransplanted jejunal loop the dogs underwent construction of an identical loop with the muscular bridge after preparation of an jejuno-ileal autotransplantation. For this purpose, all neural, lymphatic, myogenous, and connective tissue connections with the jejuno-ileum were transected except for the fully

isolated and stripped superior mesentery artery and vein at the base of the small bowel mesentery. The perivascular and adventitial tissue of these two vessels were carefully dissected away and transected under optical magnification. From here, the mesenteries to the distal duodenum and to the distal ileum were transected in radial fashion and the distal duodenum and distal ileum 5 cm from the ileo-colonic junction were also transected. At this point, the jejuno-ileum was completely isolated from any neural/lymphatic continuity with the dog except for any neural/lymphatic elements travelling within the media of the mesenteric artery and vein.

Neurally intact and autotransplanted ileal loops were prepared in a similar way.

Remie et al. (1990) described in detail the preparation of a Thiry–Vella loop in the **rat**. After laparotomy the segment to be isolated has to be located carefully. The segment should be vascularized by two or more tributary arteries. For ligation of the arcade, two ligatures are placed around the blood vessels 2–3 mm from each other. The two ligatures are tightened, the gut wall is disinfected with iodine solution, and both the vessels of the arcade and the gut wall are cut. The same procedure is performed at the other end of the segment to be isolated. This segment is laid on gauze moistened with warm saline avoiding torsion of the vessels. For end-to-end anastomosis the remaining parts of the gut are approximated and two corner sutures are placed. A continuous suture with transfixing stitches is placed on the anterior and posterior wall. Using a pair of sharp scissors two holes are made in the abdominal wall and the skin. Subsequently, standard end-to-side anastomosis technique is used to sew the gut to the internal abdominal muscle. The two stay sutures are at 180° and the posterior wall is sutured first. Following this, the skin is sutured to the abdominal muscle, using a running suture.

Chu et al. (1995) used Thiry–Vella fistulas of either the jejunum or ileum in rats in order to determine whether the trophic effects of bombesin on the small bowel mucosa are mediated by nonluminal factors or endogenous luminal secretion.

Bárdos and Nagy (1995) prepared double Thiry–Vella fistulas in rats. The first Thiry–Vella loop was created from the lower duodenum. After 1 month or more, a similar fistula was prepared from the upper part of the colon. The two loops were positioned along the midline and formed a line of 4 openings of the abdominal wall providing an easy access for inserting stimulatory devices to either of the loops in the same animal. A rubber balloon made of latex rubber was tied

to a silicon rubber tube, inserted into the isolated loop via one of its orifices and then fixed to the body by a tape. Stimulation was performed by injection of various volumes of water into the balloon from an attached syringe. Behavioral reactions of the animals were recorded as scores during 10 s stimulation periods.

In order to purify the putative luminal cholecystokinin-releasing factor, Spannagel et al. (1996) collected intestinal secretions by perfusing a modified Thiry–Vella fistula of jejunum in conscious rats.

Snoj et al. (1992) found that phosphatidylcholine inhibited postoperative adhesions after small bowel anastomosis in the rat.

Gianotti and Tchervenkov (1992) used a Thiry–Vella loop in **guinea pigs** to study the stimulatory effect of intraluminal nutriment on burned guinea pig intestinal mucosa.

Philpott et al. (1993) created Thiry–Vella loops in 21 week old **rabbits** in order to study the influence of luminal factors on intestinal repair during refeeding of malnourished infant rabbits.

Silbart et al. (1996) examined the ability of several bacterial endotoxins and their subunits to act as adjuvants or carrier proteins in stimulating an intestinal secretory Ig4 response to 2-acetylaminofluorene using Thiry–Vella loops in rabbits.

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J.4.3.10

Continuous Recording of Mechanical and Electrical Activity in the Intestine of Conscious Dogs

PURPOSE AND RATIONALE

Cyclic motor activity occurring in almost all parts of the gastrointestinal tract is due to a migrating electric complex (Szurszewski 1969; Fioramonti et al. 1980;

Sarna and Condon 1984; Sarna et al. 1984; Sarna 1985). Smooth muscle cells of the gut show periodic oscillations of membrane potentials called electrically controlled activity; they are also named slow waves, basic electrical rhythm, or pacesetter potentials. If the membrane potential depolarizes beyond a certain threshold during such an oscillation, the smooth muscle contracts. This is usually associated with a rapid burst of electrical oscillations, called electrical response activities or spikes. Electrical response activity is, therefore, associated with contractions on a 1:1 basis.

Implantation of extraluminal force transducers allows the monitoring of contractile activity in conscious animals (Bass and Wiley 1972; Ormsbee and Bass 1976; Ormsbee et al. 1981). The data can be analyzed by computer (Ehrlein and Hiesinger 1982; Schemann et al. 1985). The effects of opiate agonists, morphine, natural enkephalins, endorphins, and synthetic enkephalins and opiate antagonists, like nalorphine, as well as other gastrointestinal hormones, such as motilin, can be studied (Bickel and Belz 1985, 1988; Bickel et al. 1985).

PROCEDURE

Male Beagle dogs weighing 15–20 kg are anesthetized with thiobarbital and anesthesia is then maintained with halothane and a mixture of N₂O and O₂ (3:1). Under aseptic conditions several miniaturized strain gauge force transducers are sutured onto the muscular layers of the gastrointestinal tract. Before implantation, the transducers are externally calibrated giving linear signals over a range from 10 to 300 mN. Each transducer has its recording axis perpendicular to the longitudinal axis of the intestine to record contractions of the circular smooth muscles. The proximal ends of the strain gauge transducers are fitted to a plug embedded in a stainless-steel cannula implanted into the abdominal wall. The signals are continuously recorded and stored in a HP 9835A Hewlett Packard computer, allowing later analysis of the data.

For measuring electrical activity, bipolar electrodes are implanted at several parts of the intestinal tract. The lead wires from the electrodes are externalized via a stainless-steel cannula. Electromyogram is recorded on an electroencephalograph.

The animals are kept in a standardized environment with one daily feeding at 9:00 A.M. The dogs are trained to stand in a Pawlov stand. Eighteen hours prior to the beginning of the experiment, the animals are fasted with access to water. Motility data and electrical activity are first recorded for fasting dogs without

drug treatment. Then the drug is injected intravenously during the period of quiescence of the migrating motor complex.

EVALUATION

Recordings are analyzed for duration of cycles (min) and motor complexes (min), mean height (mN) and frequency (*n*/min). The length of the cycles is measured from the beginning of one complex to the beginning of the next complex. Electromyogram is analyzed for slow waves (cycles/min), maximum amplitude of the spike bursts (mV), duration of the spike bursts (ms) and duration of the effect (min). The data before and after administration of various doses of the test compound are compared by statistical means (Student's *t*-test).

MODIFICATIONS OF THE METHOD

Itoh et al. (1977) and Nagakura et al. (1996) used extraluminal force transducers for recording contractile motility of the gastrointestinal smooth muscle in conscious dogs.

Nakada (1995) recorded gastrointestinal and gallbladder contractions in conscious dogs by chronically implanted strain gauge transducers and gallbladder volume changes by a chronically indwelling gallbladder catheter.

Orihata and Sarna (1994) investigated contractile mechanism of gastroprokinetic agents in conscious dogs. The spatial and temporal parameters of gastric, pyloric and duodenal contractions during the entire period of gastroduodenal emptying, during a 60-min period of drug infusion and during the post-drug-infusion period were analyzed by a computer method.

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J.4.4

Absorption

J.4.4.1

Everted Sac Technique

PURPOSE AND RATIONALE

The everted sac technique is used to study the transport of substances from the mucosal to the serosal surface (Wilson and Wiseman 1954).

PROCEDURE

Male Wistar rats weighing 150–220 g are anesthetized with 30 mg/kg pentobarbitone s.c. A midline abdominal incision is made. The rats are sacrificed by cardiac puncture. The small intestine from the ligament of Treitz to the ileocaecal junction with a length of 6–7 cm is rapidly removed and everted with a glass rod. The sac is securely ligated at both ends and filled with Krebs-Henseleit bicarbonate buffer solution (pH 7.4) containing 0.4% glucose and pre-gassed with 95% O₂/5% CO₂. The sac is incubated at 37°C in a glass vessel containing the same buffer solution and gassed with 95% O₂/5% CO₂.

After 5 min, the drug solution is added to the glass vessel and the preparation is further incubated. After given time intervals, the fluid from the everted sac is removed and the volume determined. The concentration of the drug transported from the mucosal to the serosal side is determined by appropriate analytical methods.

EVALUATION

Determinations of the drug in the everted sac at different time intervals allow evaluation of pharmacokinetic parameters.

MODIFICATIONS OF THE METHOD

Madar (1983) used the small intestine everted sac of **chicken** for demonstration of amino acid and glucose transport.

Harnett et al. (1989) used everted segments of distal ileum of **rats** to study taurocholate absorption.

Turner et al. (1990) used everted sacs of ileum in **sheep** to study selenate and selenite absorption.

Goerg et al. (1992) used the stripped descending colon of the **rat** as everted sac to study the inhibition of neuronally mediated secretion in rat colonic mucosa by prostaglandin D₂.

Under ether anesthesia, the descending colon is removed and transferred into ice-cold bathing solution. The colon is placed on a plastic rod. After a circular incision with a blunt scalpel, the serosa and muscularis propria are stripped away, leaving the mucosal-submucosal preparation consisting of the mucosa, the muscularis mucosae, and a part of the submucosa with the completely preserved submucosal plexus. The tube-like mucosal-submucosal preparation is mounted as an everted sac in a holding apparatus (volume of the outer compartment 25 ml). The potential difference between the outer mucosal side and inner serosal side is measured by two agar bridges. The tissue is short-circuited by a voltage clamp after correcting for the offset potential and compensating for the solution resistance.

Moreover, transmural ion fluxes can be determined with the everted sac technique. Net fluxes of sodium, chloride, and potassium are studied by direct measurements of volume changes and changes of electrolyte concentrations in the inner volume of the everted sacs. Unidirectional fluxes can be determined simultaneously with the direct measurement of net fluxes by adding the isotopes ²²Na⁺ and ³⁶Cl⁻ to one side of the everted sac.

Schilling and Mitra (1990) used the everted gut technique in rats to study enteral insulin absorption.

Mizuma et al. (1993) studied the active absorption in the intestine and metabolism of the β - and α -anomers of the glucoside and galactoside of p-nitrophenol to find a more suitable prodrug for poorly absorbed drugs. The everted sac technique was used to investigate the intestinal absorption of these glycosides from the mucosal to the serosal side of the rat jejunum.

Kitagawa et al. (1996) investigated the influence of various factors, such as temperature, on the absorption of methochlorpromazine in the small intestinal everted sac of rats.

Tanaka et al. (1996) estimated the transport characteristics of thyrotropin-releasing hormone (TRH) and its chemically modified derivative with lauric acid (Lau-TRH) across the rat small or large intestine by means of an *in vitro* everted sac experiment.

Sasaki et al. (1995) studied the absorption characteristics of azetirelin, a new thyrotropin-releasing hormone analogue in rats by means of *in situ* closed loop and *in vitro* everted sac experiments.

Toskulkao et al. (1995) examined the effects of stevioside (a natural non-nutritive sweetening agent) and of steviol (a product of enzymatic hydrolysis of stevioside) on intestinal glucose absorption in hamster jejunum using the everted sac technique.

Motozono et al. (1994) determined the effect of age on gastrointestinal absorption of tobramycin in suckling, weanling and adult rats by an everted sac method.

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J.4.4.2

Stomach Emptying and Intestinal Absorption in Rats

PURPOSE AND RATIONALE

Reynell and Spray (1956) described a method for the simultaneous measurements of gastric emptying, intestinal transit and absorption of test substances in the rat using phenol red as marker.

PROCEDURE

Adult male Wistar rats weighing 200–300 g are allowed water but are deprived of food 24 h before the experiment. They are treated orally or subcutaneously with the test compound 15 min prior to oral administration by gavage of 1.5 ml 0.07% phenol red in 2% carboxymethylcellulose solution. Fifteen min later the animal is sacrificed and the stomach is immediately removed. The whole stomach including the stomach content is alkalized with 1 N NaOH and homogenized. The homogenate is filtered, and after precipitation of the protein with 10% trichloroacetic acid, centrifuged for 15 min at 3000 rpm. The concentration of phenol red in the supernatant is measured colorimetrically in a photometer at 546 nm.

EVALUATION

Percentage of stomach emptying (S_e) is calculated according to the following formula:

$$S_e = 100 - P_s \times P_a^{-1} \times 100$$

- P_s = Concentration of phenol red in the stomach ($\mu\text{g/ml}$)

- P_a = Concentration of phenol red in the initial solution after addition of equal volumes of 1 N NaOH and trichloroacetic acid ($\mu\text{g/ml}$)

MODIFICATIONS OF THE METHOD

By selective ligations of the different parts of the gastrointestinal tract and analysis for phenol red contents and for drug substance, as well as by sacrificing the animals after various time intervals, the degree and time course of absorption can be studied.

Droppleman et al. (1980) described a simplified method for assessing drug effects on gastric emptying in **rats**. Three ml of a semi-solid test meal, based on methylcellulose, are given to rats fasted 24 h prior to the experiment. At a specified time following the test meal, the rats are sacrificed, laparatomized, and the stomachs removed. The full stomachs are weighed on an analytical balance; they are opened and rinsed. Excess moisture is removed and the empty stomach weighed again. The difference is subtracted from the weight of 3 ml of the test meal, indicating the quantity emptied from the stomach during the test period. Gastric motor stimulants, e. g., metoclopramide increase, and anticholinergic compounds decrease gastric emptying.

Megens et al. (1990) used phenol red as marker to measure gastrointestinal propulsion after castor oil or paraffin oil challenge in **rats**.

Hedge et al. (1995) studied 5-HT₄ receptor mediated stimulation of gastric emptying in rats using a specially prepared semi-solid test meal containing charcoal.

Bonnafeous et al. (1995) investigated benzodiazepine-withdrawal-induced gastric emptying disturbances in rats. Male Wistar rats, weighing 200–250 g, fasted for 16 h, received by gavage 2 ml of a test meal containing 1 $\mu\text{Ci/ml}$ of ⁵¹Cr sodium chromate, 15 min after drug administration. Thirty min later, the animals were sacrificed by cervical dislocation. The stomach, small intestine (10 segments) and the colon were excised and placed into tubes. Radioactivity was determined by placing the tubes in a gamma counter. Gastric emptying was calculated as the percentage of total counts found in the small intestine and the colon.

Varga et al. (1995) determined gastric emptying in rats 5 min after a 3-ml intragastric load of 0.9% NaCl using phenol red as marker in order to define which bombesin receptors are involved in the delay of gastric emptying by bombesin-like peptides.

Lasheras et al. (1996) studied gastric emptying in rats. Sixty min after oral administration of vehicle or test compounds, the rats received by gavage 40 steel

spheroids (1 mm diameter) in 2 ml 3% carboxymethylcellulose. Sixty min later, the animals were sacrificed and the spheroids remaining inside the stomach counted.

Yegen et al. (1996) studied the inhibitory effects of gastrin releasing peptide on gastric emptying in rats using methyl cellulose and phenol red as non-absorbable marker.

Haga et al. (1994) studied gastric emptying in **mice**. Male mice, weighing 18–22 g, had free access to food and water before the experiment. The test compounds were administered orally in 10 ml/kg 0.5% methylcellulose solution. The mice were deprived of food and water and sacrificed 4 h later by cervical dislocation. The stomachs were removed and opened. The contents of the stomach were mixed with 10% trichloroacetic acid, and centrifuged at 3000 rpm for 30 min. The weight of the sediment was taken as the food remaining in the stomach.

Ding and Håkanson (1996) examined the effect of drugs on a cholecystokinin-A receptor-mediated response by gastric emptying of a charcoal meal in mice.

Costall et al. (1987) used the **guinea pig** to study the influence of a 5-HT₃ antagonist on gastric emptying.

Brighton et al. (1987) used scintigraphy following indium-111-labeled meals in Beagle **dogs** and **baboons**. Indium-111-labeled polystyrene beads (500 mCi per dog) were mixed into a meal consisting of 50 g of finely crushed commercial dog food and 50 ml of milk. Images of 1 min duration were taken every 5 min for a period of 1 h using a large field of view gamma camera (ON Sigma 410).

Gullikson et al. (1991, 1993) studied gastric emptying of a solid meal in dogs.

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J.4.4.3

Intestinal Drug Absorption

PURPOSE AND RATIONALE

Doluisio et al. (1969) described an *in situ* rat gut technique to determine absorption rates of drugs.

PROCEDURE

Male Sprague-Dawley albino rats weighing 220–260 g are fasted 16–24 h prior to surgery allowing, however, drinking water ad libitum. They are kept in cages having wide mesh floors to prevent coprophagia. One hour prior to surgery, the animals are anesthetized with 1 g/kg ethylurethane i.p. The small intestine is exposed by a midline abdominal incision, and two L-shaped glass cannulae are inserted through small slits at the duodenal and ileal ends. Care has to be taken to handle the small intestine gently in order to maintain an intact blood flow. The cannulae are secured by ligation with silk suture, and the intestine is returned to the abdominal cavity. Four cm long segments of tubings are attached to the exposed ends of both cannulae, and a 30-ml hypodermic syringe fitted with a three-way stop-

cock and containing perfusion fluid warmed to 37°C is attached to the duodenal cannula. As a means of clearing the gut, perfusion fluid is passed slowly through it and out the ileal cannula to be discarded until the effluent is clear. The remaining perfusion solution is carefully expelled from the intestine by means of air pumped through from the syringe.

Immediately afterwards, 10 ml of drug solution are introduced into the intestine by means of a syringe. The stopwatch is started, and the ileal cannula is connected to another 30 ml syringe fitted with a three-way stopcock. This arrangement enables the operator to pump the lumen solution into either the ileal or the duodenal syringe, remove 0.1-ml aliquots, and return the remaining solution to the intestine within 10–15 s. To assure uniform drug solution concentrations throughout the gut segment, aliquots are removed from the syringes alternatively. Samples are collected every 5 min. Depending on the drug to be studied, the concentration is determined by chemical methods.

EVALUATION

The concentrations of the drug are plotted on a logarithmic scale on the ordinate versus time on the abscissa on a linear scale. Half-life values can be calculated.

MODIFICATIONS OF THE METHOD

Ochsenfahrt (1979) described a more sophisticated method to measure the absorption of drugs in the vascularly perfused, isolated intestine of the rat. Male rats weighing about 350 g are anesthetized with urethane. The abdomen is opened by a midline incision. The mesentery of the ascending and transverse colon is gently pulled away from the mesentery of the small intestine. The vessels to the colon are ligated and cut. The superior mesenteric artery is freed from the surrounding mesentery. The duodenal vessels are ligated and cut. A suitable segment of the jejunum of about 7 cm length is selected. The proximal cannula is tied into the lumen of this segment, and the duodenum is cut. The lumen of the segment is washed with 3–4 ml warm Krebs-Henseleit solution. Then, the distal cannula is tied. After the mesenteric vessels distal to the experimental segment are ligated, the rest of the small intestine, ileum, and colon is excised. Temporarily, the intestine is covered with gauze soaked with saline solution.

The preparation of the rat is interrupted for 8–10 min, while a second rat (donor rat), which has been anesthetized with urethane, is prepared. The donor rat supplies the arterial blood; the sponta-

neous respiration is supported with a respiration pump through a tracheal cannula. The right jugular vein of the donor rat is cannulated for the blood infusion and 2 mg heparin dissolved in 0.1 saline solution is administered. A cannula is placed into the left carotid artery of the donor rat; this tube is later connected to the superior mesenteric artery of the test segment. Blood lost by the donor rat is replaced with heparinized blood taken from other rats immediately before the beginning of the experiment.

In the test animal, a thin silicon tube is inserted in the left carotid artery. Two mg heparin, dissolved in 0.1 ml saline, are injected. The superior mesenteric vein is ligated distal to the splenic vein and a plastic cannula is inserted for venous outflow. The cannula is then connected to a drop counter. The luminal cannulae of the segment are connected to a recirculation unit which is filled with Krebs-Henseleit solution at 37°C, and the luminal perfusion of the segment (mucosal solution) is started. The mucosal solution is oxygenated and recirculated. The superior mesenteric artery is ligated with a thread at its origin at the aorta and then cannulated. This cannula is first connected to the carotid artery of the test animal and later to the carotid artery of the donor animal. The isolated vascularly perfused segment is suspended in a serosal bath containing Krebs-Henseleit solution at 37°C. Venous blood outflow is collected in plastic vials at 15 min intervals. Samples are taken from the mucosal and serosal solutions at the beginning and the end of each period. By this way, the mucosal disappearance rate, the venous appearance rate, and the serosal appearance rate can be measured.

Schilling and Mitra (1992) measured insulin absorption from the distal duodenum/proximal jejunum and from the distal jejunum/proximal ileum in anesthetized rats by the closed-loop technique.

Schümann and Hunder (1996) described a modified device for the differentiated study of intestinal transfer in isolated intestinal segments from mice and suckling rats *in vitro*. A luminal perfusion system for small intestinal segments was adapted for the use in mice and rat pups to investigate longitudinal differences in drug and toxin transfer.

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J.4.5

Duodenal Ulcer Formation

J.4.5.1

Cysteamine-Induced Duodenal Ulcers in Rats

PURPOSE AND RATIONALE

Duodenal ulcers can be induced in rats by repeated administration of cysteamine or propionitrile in rats (Dzan et al. 1975; Szabo 1978; Szabo et al. 1979).

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 200 g are used. Cysteamine-HCl is administered three times on day 1 in a dose of 280 mg/kg orally. Protective drugs, such as H₂-antagonists, are given 30 min prior cysteamine treatment. The rats are sacrificed on the third day. For histological evaluation, the stomach and duodenum are fixed in 10% aqueous buffered formaldehyde and paraffin-embedded sections are stained with hematoxylin and eosin. Duodenal ulcers develop in the anterior (antimesenteric) and posterior wall of the proximal duodenum, about 2–4 mm from the pylorus. The more severe ulcers, located on the anterior wall, frequently perforate, resulting in focal or generalized peritonitis, or penetrate into the liver. The opposite ulcer invariably penetrates into the pancreas.

EVALUATION

The intensity of the duodenal ulcer is evaluated using scores from 0 to 3.

- 0 = no ulcer
- 1 = superficial mucosal erosion
- 2 = deep ulcer usually with transmural necrosis
- 3 = perforated or penetrated ulcer

CRITICAL ASSESSMENT OF THE METHOD

In view of the development of modern gastric K⁺/H⁺-ATPase inhibitors the predictive value of methods using experimental ulcers in the rat for clinical healing rates in man has been challenged (Herling and Weidmann 1994).

MODIFICATIONS OF THE METHOD

The cysteamine-induced duodenal ulcer has been used for pharmacological studies by many authors, e.g.:

Evangelista et al. (1992), Krantis et al. (1993), Pascaud et al. (1993), Tanaka et al. (1993), Morimoto et al. (1994), Pendley et al. (1995), Sikiric et al. (1997), Drago et al. (1999).

Okabe et al. (1971), Sato et al. (1989) described a method for experimental, penetrating gastric and duodenal ulcers in rats. Rats were anesthetized with ether and an incision was made in the abdomen. A round metal mold, 6 mm in diameter, was placed in close contact with the serosal surface of the duodenal wall, about 7 mm distal to the pylorus. **Glacial acetic acid** (60 ml) was poured into the mold and was left in place for 20 s. After the acetic acid was removed, the treated surface was rinsed with 50 ml of 0.02 N NaOH and the abdomen was closed. A drug or the vehicle was given p.o. once a day for 14 consecutive days beginning 2 days after the operation. The animals were sacrificed on the 16th day after the operation and the ulcerated area (mm²) was measured.

Mepirizole-induced duodenal ulcers were described by Okabe et al. (1982), Sato et al. (1989), Tanaka et al. (1989). A drug or the vehicle was given p.o. 30 min before mepirizole (200 mg/kg) was administered s.c. Twenty-four hours later, 1 ml of 0.5% Evan's blue solution was injected via the tail vein of each rat and the rats were sacrificed by CO₂ asphyxiation. The gastroduodenal region was removed and examined for lesions.

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J.4.6

Models of Inflammatory Gut Disease

J.4.6.1

Experimental Ileitis

PURPOSE AND RATIONALE

An experimental model of inflammatory bowel disease produced by the intraluminal administration of the cytotoxic plant lectin ricin into the rabbit ileum was developed by Sjogren et al. (1994), Goldhill et al. (1995, 1997). *In vitro*, electric field stimulation results

in larger non-cholinergic excitatory junction potentials in ricin-treated circular muscles than in controls.

PROCEDURE

Inflammation

Acute ileitis is induced with ricin in male New Zealand white rabbits. The animals are anesthetized with intramuscular xylazine (9 mg/kg) and ketamine (50 mg/kg) and maintained with intravenous pentobarbital (15 mg/kg). A midline incision is made, a ligated terminal ileal loop (~10 cm in length) is constructed in each animal and 1 ml of ricin (1 mg/ml) or vehicle is injected into the lumen. The loop is removed after 5 h, a time period that allows development of ileitis, abnormal myoelectric activity and increased response to electric field stimulation. Animals are sacrificed with an overdose of pentobarbital. The loop is opened along its length, gently flushed of luminal contents with cold oxygenated Krebs-bicarbonate-saline and prepared for contractility studies.

Contractility Studies

Muscle strips (~1 × 0.4 cm) with mucosa removed are cut in the axis of the circular muscle and attached to isometric tension transducers in 10-ml organ baths with modified oxygenated Krebs-bicarbonate-saline at 37.5 ± 0.5°C. Tissues are allowed to equilibrate at L_i (the length at which no tension can be measured) for 20 min. The strips are then stretched to L_0 , which is determined as the length at which maximum force is generated in response to acetylcholine (0.5–1 mM). Strips are then allowed to equilibrate for an additional 20 min.

Effect of Inflammation on Response to Tachykinins

Concentration-response curves are constructed to substance P and neurokinin agonists (or analogues) on separate muscle strips in vehicle- and ricin-treated tissues. Studies are performed in the presence or absence of tetrodotoxin to distinguish between neural and non-neural effects of ricin treatment.

Effects of Ricin Treatment to Responses to Electrical Field Stimulation

Muscle strips are passed through a pair of ring electrodes (2-mm diameter) and stimulated for 10 s by square-wave pulses (0.5 ms duration, supramaximal voltage) at 1–10 Hz. Stimulation is performed in the presence of atropine (1 mM) and N^G -nitro-L-arginine methyl ester (L-NAME) (0.1 mM).

EVALUATION

Maximum increases in muscle tone in response to tachykinin addition of electrical field stimulation are

obtained through visual analysis of chart recorder outputs. Responses to tachykinins are expressed as absolute tension development. Electrical field stimulation data are expressed as a percentage of the response to 1 μ M acetylcholine added at L_0 to reduce the variation of the data. Values are given as mean ± SEM. Tachykinin concentration responses are fitted to sigmoid curves and EC_{50} values (with 95% confidence intervals) are determined from these curves. Differences between frequency or concentration-response curves are assessed statistically by multivariate analysis of variance, with adjustments made for multiple comparisons. In cases in which curves are significantly different to one another, maximal responses were compared statistically using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Miller et al. (1991) induced ileitis in **rabbits** by luminal perfusion with histamine monochloramine or with acetic acid.

Rachmilewitz et al. (1997) induced inflammation in the small intestine in **rats** by intrajejunal administration of 0.1 ml 2% iodoacetamide.

Sukumar et al. (1997) induced ileitis in rats by two doses of indomethacin (7.5 mg/kg) administered subcutaneously 24 h apart.

Ileitis in **guinea pigs** was induced by intraluminal trinitrobenzene sulfonic acid (Miller et al. 1993; Izzo et al. 1998; Mazelin et al. 1998).

Likewise, ileitis in **hamsters** was induced by intraluminal injection of trinitrobenzene sulfonic acid (Boyd et al. 1995).

Shibata et al. (1993) induced ileitis in **dogs** by administration of 10 ml 100% ethanol and 1 g trinitrobenzene sulfonic acid dissolved in 10 ml water through a tube inserted into the ileum.

Interleukin-10 (IL-10) is produced by T cells, B cells, and macrophages. It down-regulates the function of T helper (Th) -1 cells, natural killer (NK) cells, and macrophages. In 1993, Kuhn et al. reported that, in IL-10^{-/-} mice, inflammation occurred in the whole intestine. The lesions were observed mainly in the duodenum, proximal jejunum, and ascending colon. Pathological thickening of the intestinal wall, due to hyperplastic changes, was observed in the duodenum and jejunum. In the colon, goblet cell depletion, degeneration of the epithelium, infiltration of IgA-producing plasma cells, and an increase in major histocompatibility complex (MHC) class II expression were detected. As in the IL-2^{-/-} mice, the activation of CD4⁺ cells and the depletion of their inhibitor, the regulatory T cells, are presumed to be the cause of the inflam-

mation. In 2000, Jijon et al. assessed the mechanism behind a poly (ADP-ribose) polymerase (PARP) induced increase in epithelial permeability that is associated with chronic, non-resolving colitis, which develops spontaneously in the IL-10-gene-deficient mouse. They demonstrated that treatment of IL-10-deficient mice with the PARP inhibitor 3-aminobenzamide reversed the typical signs of Crohn's disease in these mice, namely increased permeability, high levels of mucosal interferon- α (IFN- α) and tumor necrosis factor α (TNF- α), and increased nitric oxide (NO) production. In the same year, Cantorna et al. (2000) used the IL-10-deficient mouse model to investigate the role of vitamin D on the course of inflammatory bowel disease (IBD). The animals were divided into three groups: vitamin D deficient, vitamin D sufficient, and active vitamin D supplemented. In contrast with vitamin D-deficient IL-10 knockout (KO) mice, vitamin D-sufficient mice did not develop diarrhea, waste, or die prematurely. To the authors' surprise, supplementation with active vitamin D for only 2 weeks blocked the progression and significantly ameliorated the symptoms in the mice that had established spontaneously developed IBD. The importance of animal models for the evaluation of pharmacological strategies was further emphasized in a straightforward ministudy conducted by Gratz et al. (2002), who administered murine monoclonal anti-TNF- α antibodies intraperitoneally into IL-10 KO mice that had established IBD. Demonstration of significant histological improvement of inflammation that correlated well with a resolution of diarrhea and rectal bleeding accentuated the pivotal role that TNF- α appears to play in the pathogenesis of IBD and emphasized the importance of this model. Recently, a pioneer therapeutic approach to IBD was tested in the IL-10 KO model by Watanabe et al. (2003a, 2003b). The group developed poly-DL-lactic acid microspheres containing dichloromethylene diphosphonate that, once administered rectally, were specifically taken up by macrophages, subsequently depleting them. The authors showed reduced numbers of resident macrophages in the intestinal lymphoid follicles in this model, associated with suppression of development of chronic colitis.

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J.4.6.2

Experimental Colitis

PURPOSE AND RATIONALE

Inflammatory bowel diseases, ulcerative colitis and Crohn's disease, represent chronic alteration of the

gastrointestinal tract of unknown etiology perhaps involving immunological events. The immunological parameters have been described as secondary but may possibly be attributed to the chronicity of the disease. Several compounds have been described to elicit cell-mediated immune responses in the gut, such as dinitrochlorobenzene (DNCB) (Rosenberg and Fischer 1964; Norris et al. 1982; Norris 1989) or 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Morris et al. 1989; Selve and Wöhrmann 1992).

PROCEDURE

A three-step concept is realized to mimic the human disease, using 2,4,6-trinitrobenzene sulfonic acid (TNBS) as a defined hapten:

1. specific hypersensitivity by active immunization,
2. local inflammation by local challenge,
3. chronicity by chronic application of the immunogen.

Female Sprague Dawley rats weighing 150–200 g are sensitized by intradermal injection of 0.8% TNBS (Fluka, Neu-Ulm, Germany) solution into a shaved area on the back once daily for three consecutive days. TNBS is dissolved in 0.05 ml Freund's incomplete adjuvant together with 1 mg/ml ovalbumin. After 18 days, the animals receive a further intradermal booster injection. Intradermal challenge of 0.08% TNBS in 0.05 ml 0.9% NaCl solution with or without ovalbumin, or ovalbumin solution without TNBS, is given 14 days later in order to determine the type and specificity of the immunological reaction.

Ten days after the intradermal challenge, a flexible polyethylene tube of 0.5 mm diameter is implanted under ketamine (100 mg/kg i.p.) anesthesia 15 cm proximal to the cecum and emerging at the neck for TNBS or drug administration. After a 10-day recovery phase, the animals are treated daily for 3 weeks with 0.08% TNBS in saline (0.2 mg/rat) given through the catheter. Control groups receive only saline. Drugs are applied either by gavage twice a day, suspended in carboxymethyl cellulose, or intraluminally once a day, suspended in saline. The animals are sacrificed by CO₂ inhalation 24 h after the last intraluminal application of TNBS. The distal 10 cm of small intestine anterior to the ileo-caeco-colic junction (5 cm distance to the open end of the catheter) including Peyer's patches are dissected, cut open longitudinally and rinsed with saline.

Immediately after dissection, the distal small intestine is visually assessed for inflammation according to the following scores:

| <i>Enteritis score</i> | <i>Gross morphology</i> |
|------------------------|--------------------------------------------------------------------------------------------|
| 0 | No visible damage of the whole 10 cm the small intestine |
| 1 | Slight inflammation, slight redness (hyperemia), villi visible under 15 fold magnification |
| 2 | Intermediate inflammation, discontinuous hyperemia, intermediate redness of villi |
| 3 | Intensive inflammation, intensive hyperemia, intensive redness of villi |

The dissected gut segments, precisely 10 cm long, are weighed for measurement of edema formation. They are then incubated in Tris buffer for 30 min at 37°C in a shaking water bath (1 ml/100 mg tissue, Tris 50 mM, pH 7.4, 100 mM NaCl, 1 mM CaCl₂, 1 mg/ml glucose). Following incubation, aliquots of the solutions are centrifuged (13,000 g, 2 min, 20°C) for determination of leucotriene B₄ (TB₄) by a commercial radioimmuno-assay (e. g., Amersham Buchler, Brunswick).

EVALUATION

Results are expressed as means ± SEM of (*n*) experiments. Differences between control and inflamed tissue, and influence of drug treatment are compared. Statistical significance is calculated by Wilcoxon-Mann-Whitney *U*-test for unpaired data. The level of significance is taken as $p < 0.05$.

MODIFICATIONS OF THE METHOD

Several authors used the trinitrobenzene sulfonic acid model in *rats* with slight modifications (Hogaboam et al. 1996; Kitano et al. 1996; Yue et al. 1996; Lora et al. 1997; Taniguchi et al. 1997; Cruz et al. 1998; Fries et al. 1998; Goldhill et al. 1998).

Zea-Ariate et al. (1994) studied chronic colitis in Wistar rats after intracolonic instillation of 20 or 42 mg of trinitrobenzene sulfonic acid in 30% and 40% ethanol and compared the effect with the administration of ethanol alone.

Alternatives to the 2,4,6-trinitrobenzene sulfonic acid model in rats were proposed:

Wallace et al. (1995), Hawkins et al. (1997) recommended dinitrobenzene sulfonic acid to produce experimental colitis in the rat.

Patterson and Colony (1983) tested sulphasalazine and 5-aminosalicylic acid in experimental colitis induced in **guinea pigs** by topical dinitrochlorobenzene.

Neurath et al. (1996) studied chronic intestinal inflammation induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in **mice**.

These authors (Neurath et al. 1997) investigated the role of tumor necrosis factor in this mouse model and showed that no significant TNBS-induced colitis could be induced in mice in which the TNF-alpha gene had been inactivated by homologous recombination.

Cuzzocrea et al. (2004) found that 5-aminoisoquinolinone reduces colon injury in experimental colitis induced by dinitrobenzene sulfonic acid in mice.

Antony et al. (1997), Oyen et al. (1997) used trinitrobenzene sulfonic acid to induce experimental colitis in **rabbits**.

Dams et al. (1998) compared gamma-camera imaging in rabbits with colitis after retrograde instillation of trinitrobenzene sulfonic acid with technetium-99m-labeled liposomes after various time intervals with macroscopically scored severity of inflammation.

A model of diffuse colitis in **rats** induced by intraluminal colonic instillation or serosal application of dilute **acetic acid** was described by MacPharson and Pfeiffer (1976, 1978). Ritzpatrick et al. (1990) tested the anti-inflammatory effects of various drugs on acetic acid induced colitis in the rat.

For instillation into the colon of rats, a 2 cm length of soft polyethylene tubing is fitted to a Luer stub adapter. The open end of the tubing is sealed with glue. The whole length of the tubing is perforated with a needle at 0.5 mm length by four holes, 90° apart. A 1 ml syringe containing 10% acetic acid in saline is fitted to the adapter. The tubing is inserted intrarectally into the colon of rats and 0.2 ml is injected into the lumen. After 10 s contact *in situ*, the remaining acid is withdrawn and the lumen washed with three successive 0.5 ml volumes of isotonic saline. Test drugs are administered daily during the following days. Diffuse colonic lesions appear after 3 days in control animals and the animals experience a bloody diarrhea. An initial mucosal inflammation develops into submucosal edema; petechial hemorrhages become enlarged with subsequent neutrophil invasion, and pseudopolyps become evident.

Terzioglu et al. (1997) studied the effect of prostaglandin E₁ on the experimental colitis induced by rectal instillation of 10% acetic acid in rats.

Fabia et al. (1994) induced colitis in rats in an exteriorized colonic segment by administration of 4% acetic acid for 15 s. Four days later, this colonic segment was examined using a morphological scoring system, and measurements of myeloperoxidase activity and plasma exudation into the colonic segment.

Eliakim et al. (1995) demonstrated that ketotifen ameliorated capsaicin-augmented acetic acid-induced

colitis. Rats were pretreated with subcutaneous injections of 20, 30, and 50 mg/kg capsaicin. Colitis was induced 2 weeks later by flushing 2 ml 5% acetic acid into the proximal colon.

Higa et al. (1997) studied the role of neutrophils in the pathogenesis of acetic acid-induced colitis in mice.

Millar et al. (1996) evaluated the antioxidant potential of new treatments for inflammatory bowel disease using acetic acid-induced colitis in rats.

Myers et al. (1997) determined colonic transit in rats by calculating the geometric center of distribution of a radiolabeled marker (⁵¹Cr) instilled into the proximal colon after induction of distal colitis by intracolonic administration of 4% acetic acid.

Palmen et al. (1998) studied the effects of local butesonide treatment on cell-mediated immune response in acute and relapsing colitis in rats.

Several **other agents** can induce experimental colitis in animals (Kim and Berstadt 1992), such as

- **phorbol esters** (Fretland et al. 1990),
- **carrageenan** (Marcus and Watt 1969; Benitz et al. 1973; Watt and Marcus 1973; Abraham et al. 1974; Jensen et al. 1984; Kitano et al. 1994; Pricolo et al. 1996),
- **amylopectin-sulfate** (Watt and Marcus 1972),
- **dextran sulfate** (Ohkusa 1985; Okayasu et al. 1990; Axelsson et al. 1966; Shintani et al. 1997; Kanauchi et al. 1998),
- the **chemotactic peptide FMLP** (Magnusson et al. 1985; von Ritter et al. 1988; LeDuc and Nast 1990).

Ekstrom (1998) described **oxazolone**-induced colitis in rats. Dark Agouti rats were skin-sensitized with oxazolone and further challenged intra-rectally with oxazolone dissolved in carmellose sodium/peanut oil.

Suzuki et al. (1997) developed a rat model for human ulcerative colitis by using **1-hydroxy-anthraquinone** to cause severe inflammation of colonic mucosa.

Aiko et al. (1997), Satdnicki et al. (1998) induced chronic granulomatous colitis in female Lewis rats via intramural (subserosal) injections of **peptidoglycan-polysaccharide** into the distal colon.

Surfactants being used to enhance drug absorption may cause intestinal damage. Oberle et al. (1995) evaluated mucosal damage by surfactants in a single-pass *in situ* perfusion model in the rat. The release of LDH and mucus into the lumen of jejunum and colon following perfusion of the nonionic surfactants Tween 80 and Triton X-100 was determined.

Axelsson and Ahlstedt (1993) reviewed the actions of sulfasalazine and analogues in various animal models of experimental colitis, in particular in the hapten-, immune complex- and dextran models.

Bach et al. (1985) studied the inhibition of LTC synthetase and of rat liver glutathione *S*-transferases by sulfasalazine.

Stein et al. (1993) determined arachidonic acid oxidation and damage in the colon in rats stressed by the **cold-restraint** method.

Kirsner et al. (1959), Kraft et al. (1963) induced severe colitis in **rabbits** which had previously been sensitized to egg albumin and had mild colonic inflammation induced by intrarectal instillation of a small amount of diluted **formalin**.

Meenan et al. (1996) induced immune-complex colitis in rabbits by using various formalin concentrations (2%, 0.75%, and 0.5%).

Hodgson et al. (1978) induced colitis in rabbits by a modified technique. Preformed immune complex of human serum albumin and anti-HSA with antigen excess was injected to non-sensitized rabbits after provocation of mild inflammation in the colon with diluted formalin.

Kuroe et al. (1996a, b) induced granulomatous enterocolitis in rabbits by repeated submucosal injection of **muramyl dipeptide** emulsion into the rectum and colon with a flexible endoscope. Extraintestinal manifestations, such as pericholangitis, were observed.

Walsh and Zeitlin (1987) studied the effects of salazopyrin, 5-aminosalicylic acid and prednisolone on immune complex-mediated colitis in **mice**.

A survey on various genetic and immune manipulations which lead to inflammatory bowel disease in mice was given by Powrie and Leach (1995).

CD4⁺ T lymphocytes injected into severe immunodeficient (SCID) mice lead to an inflammatory and lethal bowel disease (Claesson et al. 1996; Leach et al. 1996; Rudolphi et al. 1996; Bregenholt et al. 1998).

Hermiston and Gordon (1995) described an inflammatory bowel disease in mice expressing a dominant negative N-cadherin resembling Crohn's disease.

Watanabe et al. (1998) reported that interleukin7 transgenic mice develop chronic colitis with decreased interleukin7 protein accumulation in the colonic mucosa.

Mitchell and Turk (1990) described a model of **granulomatous bowel disease in guinea pigs**. Epithelioid cell granulomas and primary macrophage granulomas were induced by the inoculation of BCG (Pas-

teur) and irradiated *Mycobacterium leprae*, respectively, into the terminal ileum.

Wallace et al. (1998) induced colitis in guinea pigs and in rats by intracolonic administration of trinitrobenzene sulfonic acid.

Some **monkeys**, such as the cotton-top tamarin, *Saguinus oedipus* (Chalifoux and Bronson 1981; Madara et al. 1985; Lushbach et al. 1985; Podolsky et al. 1988; Hesterberg et al. 1996; Warren 1996), and juvenile rhesus monkeys (Adler et al. 1990), develop **spontaneous colitis**.

CRITICAL ASSESSMENT OF THE METHODS

The relevance of animal models for the pathogenesis and treatment of human inflammatory bowel disease was reviewed by Dieleman et al. (1997) and by Sartor (1997).

A critical review of *in vitro* models in inflammatory bowel disease was given by McKay et al. (1997).

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J.5 Emetic and Anti-Emetic Activity

J.5.0.1

Assessment of Emetic and Anti-Emetic Activity in Dogs

PURPOSE AND RATIONALE

Emesis comparable to man occurs only in a few animal species. Among laboratory animals, the dog is a suitable species to test anti-emetic drugs. Apomorphine-induced emesis is also used to evaluate neuroleptic drugs (see E.5.3.8). Burkman (1982) described a technique relying upon the use of apomorphine either as a reference standard against which other emetics can be compared, or as a challenging agent against which anti-emetic compounds can be evaluated.

PROCEDURE

Beagle dogs weighing between 15 and 20 kg are used. Each dog is given 200 g food 30 min prior to an assay session. The threshold emetic dose of apomorphine hydrochloride is established for each dog by administering single doses at 5 day intervals in gradually increasing amounts. The starting dose is 0.07 mM/kg (22 mg/kg) body weight, i.m., and is subsequently increased (or decreased) as required. Injection sites alternate between contralateral gluteus muscles. After every third or fourth dose of the emetic, the animals receive an equivalent volume of vehicle under similar conditions in order to detect the presence of a conditioned emetic response.

The threshold dose is defined as the concentration provoking an emetic episode and determined for each

individual animal. The threshold emetic dose is relatively stable for a given group of dogs over a period of 2 months. Continued administration to the same dogs for longer periods of time is inadvisable as Pavlovian emetic conditioning becomes evident after 8–10 doses of apomorphine. Establishment of an emetic threshold for a test compound using a similar dosing schedule allows to quantitatively express the test compound's emetic potency as a ratio compared with the reference standard. Usually, 4–6 animals are sufficient to provide a reliable estimate of the test compound's emetic efficacy and potency.

In the anti-emetic assay, dogs whose apomorphine threshold emetic dose has been determined receive various concentrations of the potential anti-emetic drug at a given time interval prior to apomorphine. The dose initially selected for the anti-emetic is a fraction of the acute LD_{50} of this drug in mice. A new threshold dose is estimated in the presence of the test anti-emetic and compared to the threshold dose in the presence of the reference standard chlorpromazine.

EVALUATION

Using the threshold doses, the relative potency of an emetic compared to apomorphine, or the relative potency of an anti-emetic compared to chlorpromazine, is calculated.

MODIFICATIONS OF THE METHOD

Cisplatin-induced emesis in the **dog**, as described by Gylys et al. (1979), was used by Turconi et al. (1991) to test the anti-emetic properties of 5-HT₃ receptor antagonists.

Heaslip and Evans (1995) studied the emetic, central nervous system, and pulmonary activities of rolipram in the dog after i.v. and intragastral application.

Gupta and Sharma (1996) tested the activity of antioxidants against emesis induced by an intravenous dose of 3 mg/kg cisplatin in healthy mongrel dogs.

Szelenyi et al. (1994) described emesis in **domestic pigs** as a new experimental tool for detection of anti-emetic drugs and for evaluation of the emetogenic potential of new anticancer agents. Healthy young, 12–15-week-old domestic pigs of either sex were lightly anesthetized with ketamine (10 mg/kg, i.v.) and xylazine (2 mg/kg, i.v.) and a cannula inserted into a superficial vein of one of the extremities. The challenging agents, e. g., cisplatin, carboplatin, cyclophosphamide, or ifosfamide were infused intravenously at different doses during a total administration time of 15 min. After removing of the cannula the animals

were placed in their boxes for observation of emesis over 24 h. Drugs with anti-emetic potential were given intravenously 15 min prior cisplatin infusion.

Göthert et al. (1995) found a dose-dependent inhibition of cisplatin-induced emesis in pigs by anpirtoline, a mixed 5-HT₁ receptor agonist/5-HT₃ receptor antagonist.

Gardner et al. (1995, 1998) used the **house musk shrew** (*Suncus murinus*) to test antagonism against cisplatin-induced emesis.

Kwiatkowska et al. (2004) performed a comparative analysis of the potential of cannabinoids and ondansetron to suppress cisplatin-induced emesis in *Suncus murinus* (house musk shrew).

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J.5.0.2

Anti-Emetic Activity in Ferrets

PURPOSE AND RATIONALE

The ferret is a well established animal model of emesis which responds to cancer chemotherapeutic agents in a manner similar to that observed in man (Florczyk et al. 1982). The animals react with vomiting and retching after challenge with central (loperamide and apomorphine), peripheral (CuSO₄), or mixed central and peripheral (ipecacuanha, cisplatin) emetic stimuli. The model has been used to test the anti-emetic properties of 5-HT₃ receptor antagonists and tachykinin NK₁ receptor antagonists.

PROCEDURE

Adult male ferrets weighing 1 to 1.5 kg are randomly assigned to the different treatment groups. Each animal is anesthetized by inhalation with methoxyflurane. A jugular vein is cannulated and exteriorized from the outside of the neck. Following recovery from the anesthesia, the animals are dosed with the test drug or the standard or the vehicle 30 min prior i.v. administration of 10 mg/kg cisplatin. The numbers of retches and vomits occurring following the administration of the emetogen are recorded in each animal for 5 h. Retching is defined as rhythmic inspiratory movements against a closed glottis, and vomiting as forced expulsion of upper gastrointestinal contents.

EVALUATION

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls.

Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's LSD multiple comparison test.

MODIFICATIONS OF THE METHOD

Fink-Jensen et al. (1992) reported that the excitatory amino acid receptor antagonists, 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(f)quinoxaline (NBQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which preferentially block non-NMDA subtypes of excitatory amino acid receptors, effectively inhibit cisplatin-induced emesis in ferrets.

Emesis in ferrets was induced by X-irradiation or oral doses of copper sulfate (Andrews and Bhandari 1993).

Watson et al. (1995) studied the anti-emetic effects of a selective NK₁ receptor antagonist using the gag reflex in ferrets. The gag reflex is mediated by mechanoreceptors in the superior laryngeal nerve, which projects to the nucleus tractus solitarius. (Mifflin 1993). The gag reflex was evoked in conscious ferrets by gentle tactile stimulation of the pharynx and larynx and was recorded as an all or none response before and after drug administration.

Furthermore, the authors induced retching in the ferret by electrical stimulation of the vagal afferents under urethane anesthesia (Andrews et al. 1990). The dorsal or ventral abdominal vagus was isolated and ligated and the central cut-end stimulated before and after drug administration.

Duplantier et al. (1996) studied the emetic behavior in the ferret induced by inhibitors of phosphodiesterase type IV (PDE IV) and by rolipram comparing with the [³H]rolipram binding activity. Robichaud et al. (1999, 2001) compared several PDE IV inhibitors and studied the mode of action inducing emesis in ferrets.

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J.5.0.3

Assessment of Emetic and Anti-Emetic Activity in Pigeons

PURPOSE AND RATIONALE

Emesis in pigeons can be induced by various agents. Formerly, the phenomenon has been used for standardization of cardiac glycosides (Hanzlik 1929). More re-

cently, dose response curves of emesis have been determined for various agents and anti-emetic effects were evaluated (Wolff and Leander 1994, 1995).

PROCEDURE

Male White Carneaux pigeons are kept in individual stainless steel cages at constant temperature and humidity. They are maintained at 90% of their free-feeding body weights by once-daily feeding of approximately 20 g Purina Pigeon Checkers.

All testing is conducted during the illuminates phase of the light-dark cycle. On test days, the birds are fed 5 min before the start of an emetic trial. If vomiting occurs, the pigeons are given an additional 20 g of feed before being returned to their home cages at the conclusion of the observation period. Individual subjects are allowed a recovery period of at least 3 days between each drug test.

For the following compounds emetic doses are reported:

- Cisplatin 10 mg/kg, injected into a wing vein.,
- Ipecac syrup, 1 to 3 ml/kg, administered via a feeding needle passed through the crop to the opening of the proventriculus,
- emetine, 1 to 20 mg/kg, injected into the pectoralis muscle,
- m-(chlorphenyl)-biguanide (mCBG), 0.32 to 5 mg/kg, injected intramuscularly,
- ditolyganidine (DTG), 5.6 mg/kg, injected intramuscularly.

Test substances with potential anti-emetic activity are injected at various doses 15 min before the emetic challenge. The animals are observed for vomiting during 2 h.

EVALUATION

ED_{50} values for with 95% confidence limits are calculated for the activity of emetic substances, as well as for the inhibition of emesis by anti-emetic drugs after a high dose of the emetic compound.

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J.5.0.4

Activity Against Motion-Induced Emesis

PURPOSE AND RATIONALE

The house musk shrew (*Suncus murinus*) is a small insectivore that has been shown to exhibit emesis when exposed to linear reciprocation motion (Ueno et al. 1988; Okada et al. 1994).

PROCEDURE

Adult male (body weight range 55–90 g) and female (body weight range 35–50 g) are used. The animals receive a dose of the test drug or vehicle in a volume of 4 ml/kg 15 min before motion testing. The animals are placed in a Perspex chamber (11 cm wide × 22 cm long × 11 cm high) that is attached to the platform of a shaker set to execute a linear horizontal movement of 4 cm at a frequency of 1 Hz along the long axis of the chamber. The animals are allowed approximately 3 min to become accustomed to the chamber before exposure to motion for a period of 5 min, during which the number and timing of emetic episodes are recorded. An emetic episode usually consists of a short period of rapid retching followed by a vomit. A cross-over design is used for the experiment, with animals exposed to motion testing following treatment with vehicle control on one occasion, and following treatment with test drug on another. An interval of 12 days is allowed between treatments.

EVALUATION

Group results are expressed as mean ± SEM. Either Student's *t*-test or the Wilcoxon signed rank test is used as a measure of significance.

MODIFICATIONS OF THE METHOD

Gardner and Perren (1998) described a model of post-anesthesia-induced emesis in *Suncus murinus*.

Parker et al. (2004) tested the effect of cannabinoids on lithium-induced vomiting in the *Suncus murinus* (house musk shrew).

Lucot (1989) used cats to test the activity of HT₃ antagonists against motion-induced sickness.

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J.5.0.5

Foot Tapping in Gerbils

PURPOSE AND RATIONALE

Foot tapping in gerbils, a centrally mediated behavior (Graham et al. 1993; Bristow and Young 1994; Rupniak and Williams 1994; Vassout et al. (1994), has been claimed to be highly predictive for NK₁ antagonists to prevent cisplatin-induced retching in ferrets and to be a simple *in vivo* assay for CNS penetration (Rupniak et al. 1997).

PROCEDURE

Mongolian gerbils of either sex weighing 35–70 g are anesthetized by inhalation of an isoflurane/oxygen mixture to permit exposure of the jugular vein through a skin incision in the neck, using blunt dissection to clear surrounding salivary gland and connective tissues. Test compounds or vehicle are administered using an injection volume of 5 ml/kg i.v. The wound is closed and a second incision is made in the midline of the scalp to expose the skull. The highly selective, peptidase-resistant NK₁ receptor agonist GR73632 (D-Ala-[D-Pro⁹,Me-Leu⁸]substance P-(1–17) (Hagan et al. 1991) is infused directly into the cerebral ventricles (3 pmol in 5 µl i.c.v.) by vertical insertion of a cuffed 27-gauge needle to a depth of 4.5 mm below bregma. The scalp incision is closed and the an-

imal allowed to recover from anesthesia in a clear Perspex observation box (25 × 20 × 20 cm). The duration of hind foot stepping is then recorded continuously for 5 min using a stopclock. The time relapse from induction to recovery from anesthesia, with intervening i.v. and i.c.v. injections is about 3–4 min.

EVALUATION

Data are subjected to one-way analysis of variance (ANOVA), followed by Dunnett's or Newman-Keuls multiple comparison *t*-tests.

CRITICAL ASSESSMENT OF THE METHOD

The specificity of the method to predict anti-emetic activity has to be proven.

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J.6

Gall Bladder Functions

J.6.1

Bile Secretion

J.6.1.1

Cholagogic Activity in Mice

PURPOSE AND RATIONALE

A rapid method for standardization of cholagogues in mice by simple weighing the gall bladder filled with bile was published by Litvinchuk (1976).

PROCEDURE

Groups of 10 female mice (e. g., NMRI strain) weighing 15–20 g are used. Food, but not water, is withdrawn 24 h prior to the experiment. The test compound or the control solution is administered subcutaneously or

orally. After 1 h, the animals are sacrificed and bled from the carotid artery. Laparotomy is performed, the liver exposed, and a No. 75 silk ligature is tied around the cystic duct, which is detached from the bile ducts and removed from the peritoneal cavity. If a large volume of bile has been accumulated, the full gall bladder is removed together with the bile ducts. The isolated gall bladder is weighed on a suitable balance, after which the contents are removed, the gall bladder walls are washed with distilled water, dried on filter paper, and the organ is weighed again. The difference in weight of the full and the empty gall bladder indicates the quantity of bile secreted during a measured time. The concentration of cholates, bilirubin, and cholesterol in the bile can be determined.

EVALUATION

The average of secreted bile in groups of 10 treated mice is compared with the average value of the control group using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The method has the clear advantage of simplicity but does not measure the true bile excretion since the outflow from the bile bladder during the test period is neglected.

MODIFICATIONS OF THE METHOD

Sterczek et al. (1996) studied the effect of cholagogues on the volume of the gallbladder in healthy dogs fasted for 24 h by two-dimensional ultrasonography. The volume was measured immediately before the administration of each test substance and at 10-min intervals for 120 min thereafter.

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can be used as a suitable model to measure choleresis, i. e. bile production.

PROCEDURE

Male rats (e. g. Sprague-Dawley strain) weighing 300–500 g are used. Food, but not water, is withdrawn 18 h prior to the experiment. The animals are anesthetized with 5 ml/kg 25% urethane solution. The trachea is cannulated and the abdomen opened by a midline incision. The pylorus is ligated and the bile duct cannulated from the duodenum with a thin (0.05 mm diameter) polyethylene catheter which is pushed up to the liver. The secreted bile volume is measured for 30 min intervals and then the bile is returned to the duodenum. After a preperiod of 60 min, the test substances are administered subcutaneously or intraduodenally. The bile volume is registered in 30 min intervals for 2 h.

EVALUATION

The average values of the post-drug periods are compared with the pre-drug readings.

MODIFICATIONS OF THE METHOD

Several authors tested the choleric activity of plant extracts and essential oils (De la Puerta et al. 1993; Peana et al. 1994; Trabace et al. 1994) and of synthetic compounds (Grella et al. 1992; Paglietti et al. 1994) in rats.

Tripodi et al. (1993) investigated the anticholelithogenic and choleric activities of taurohyodeoxycholic acid by measurement of biliary flow and biliary solids content in rats.

Bouchard et al. (1993) induced cholestasis in rats by treatment with 17- α -ethinyl estradiol and studied the influence of oral treatment with ursodeoxycholic and tauroursodeoxycholic acids.

Vahlensieck et al. (1995) studied the effect of *Chelidonium majus* herb extract on choleresis in the isolated perfused rat liver.

Miki et al. (1993) investigated the metabolism and the choleric activity of homochenodeoxycholic acid in **hamsters** with bile fistula.

Pesson et al. (1959) recommended the **guinea pig** as the best choice among the common laboratory animals to study choleric agents.

Cohen et al. (1992) reported a study in male **black-tailed prairie dogs** (*Cynomys ludovicianus*) weighing 1.0 ± 0.2 kg anesthetized with 20 mg/kg xylazine i.m. and 20 min later with 100 mg/kg ketamine i.m. Through an abdominal incision, the cystic duct is ligated, and gallbladder bile is aspirated. A PE-

J.6.1.2

Choleric Activity in Rats

PURPOSE AND RATIONALE

In contrast to other animals, rats do not possess a bile bladder. Therefore, cannulation of the bile duct in rats

50 polyethylene cannula is inserted into the common bile duct and secured with silk sutures, thereby completely diverting bile flow for collection. The bile duct cannula is externalized, the abdominal incision closed, and the prairie dog placed in a restraining cage with access to food and water.

Matsumura et al. (1996) analyzed hypercholerisis in **dogs** with pigment gallstones after cholate infusion.

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as the surgical intervention itself may profoundly influence the results. Therefore, Remie et al. (1990, 1991) developed a technique for a permanent double bile fistula in rats. The procedure is described in detail.

PROCEDURE

Preparation of Cannulae

Cannulae are made of silicon rubber. The proximal bile cannula, which will be inserted into the common bile duct in the direction to the liver, is 18 cm long (Silastic tubing, Dow Corning, no. 605–135; 0.51 i.d. and 0.94 o.d.) and has one square cut and one bevelled end. Two silicon rings are wrapped around the cannula at 7 mm and 50 mm, respectively, from the bevelled end.

The distal bile cannula, which will be inserted into the common bile duct in the direction of the gut, is made of the same material, is also 18 cm long and has one square cut and one bevelled end. This cannula, however must have a smaller tip-diameter (Silastic tubing, Dow Corning, no. 605–105; 0.31 i.d. and 0.64 o.d.). To serve this purpose, the square cut end of the cannula is immersed in ether, causing the tubing to dilate. When the tubing is wide enough, a 13 mm piece of small diameter Silastic tubing is inserted. Subsequently, two silicon rings are wrapped around the cannula, one at the joint of the two tubes and the other 5 cm from the tip. The tip is then cut at a 45° angle, 7 mm from the first silicon ring.

The duodenal cannula (Silastic tubing, Dow Corning, no. 605–135) is also 18 cm long, and has one square and one bevelled end. An additional ring is placed 30 mm from the tip. Before the cannulae are fixed to the skull, they must be connected to a stainless steel needle bent in a 90° angle.

Anesthesia

The animal is anesthetized with halothane/N₂O/O₂.

Preparation of the Crown of the Head

The head of the animal is shaved and disinfected. An incision of about 1 cm is made and the bregma exposed. Three stainless steel screws (1.0 × 4.2 mm) are mounted in the crown, two in the left and one on the right side of the bregma. The screws are tightened that approximately 2 mm is left between the skull and the head of the screws.

Double Cannulation of the Bile Duct

The abdominal wall is shaved and disinfected and the animal secured on the operation board with adhesive tape. A midline incision is made from the level of the

J.6.1.3

Chronic Bile Fistula in Rats

PURPOSE AND RATIONALE

Most of the techniques for collection of bile in rats use restrained or anesthetized animals. Such factors as well

pubic bones to the xiphoid cartilage. The abdomen is then opened by making an incision over the linea alba towards the sternum up to the distal part of the fourth sternebra, thus exposing the xiphoid cartilage.

Then, the intestines are lifted out and are laid next to the animal on moistened gauze. Using jewelers forceps, the bile duct is stripped off its surrounding tissue and ligated with a 7-0 suture. The duct is placed under tension with an artery forceps for cannulation. With the aid of a microscope, a V-shaped hole is made just cranial of the first ligature with iridectomy scissors. The sterile proximal cannula is inserted into the duct. The second ligature is tied and pulled tight ensuring that the cannula is not obstructed. The bile is now flowing into the cannula. The first ligature is released and the threads are tied behind the silicon ring. The rat is then turned and the ligature re-clamped, thereby putting the distal part of the duct under tension. A third ligature is loosely introduced around the duct, distal to the first ligature. Another V-shaped aperture is made between the first and third ligature for insertion of the distal bile cannula. The third ligature is tied and pulled tight. The first ligature is released from the artery forceps and tied around the second cannula behind the silicon ring. All the loose threads are cut close to the knots. The sections of the cannulae which lie between the silicon rings are placed kink-free in the abdominal cavity. The cannulae are fixed using 7-0 silk suture to the abdominal muscle near the xiphoid cartilage.

Cannulation of the Duodenum

After location of the place where the bile duct enters the duodenum (sphincter of Oddi), a four fine-stitch purse-string suture (7-0) is made in the wall of the duodenum at the outer border at about 1 cm proximal to the sphincter. Using a 20G needle, an incision is made inside the purse string. The cannula is inserted into the duodenum until the first, smaller silicon ring has entered the lumen, and the purse string is tightened between the first and the second ring. This cannula together with the bile cannula is placed kink-free in the abdominal cavity and anchored to the internal muscle.

The abdomen is closed of resorbable sutures leaving 1 cm of the skin unclosed.

Subcutaneous Tunneling and Anchoring of the Cannulae

>From the back of the neck, a slender needle holder is pushed subcutaneously through the connective tissue in caudal direction as near as possible to the skin down to the xiphoid cartilage. The cannulae are then grasped and pulled through to emerge at the crown of the head. The abdominal wall is closed completely.

With a 5 cm piece of polyethylene tubing (0.75 × 1.45 mm), the two long ends of the L-shaped stainless steel adapters are connected and the short ends inserted into the respective cannulae. The cannulae together with the tubing are fixed to the skull with acrylic glue flowing under the heads of the screws.

Postoperative Care

The animals are allowed to recover in a warm and quiet place. They reach usually preoperative weight within 2-3 days, and display normal feeding and drinking behavior. Supplementation with saline besides the normal tap water may be necessary.

Collection of Bile

The animals are housed in individual metabolic cages. For bile collection, they are attached to long swiveled PE-cannulae (0.75 × 1.45 mm). A stainless steel coil is used to protect the rats from gnawing on the tubing. For continuous collection of bile, the cannula can be connected to a fraction collector.

CRITICAL ASSESSMENT OF THE METHOD

Among other applications, the method is suited to study the enterohepatic circulation of compounds.

MODIFICATIONS OF THE METHOD

Castilho et al. (1990) studied the intestinal mucosal cholesterol synthesis in rats using a chronic bile duct-ureter fistula model. Male Wistar rats weighing 300-350 g were anesthetized with 50 mg/kg pentobarbital i.p. and submitted to a bile duct-right ureter fistula utilizing a PE-50 catheter after a right-kidney nephrectomy.

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J.6.1.4

Chronic Bile Fistula in Dogs

PURPOSE AND RATIONALE

Herrera et al. (1968) described a special cannula which can be used to obtain bile or pancreatic juice from a duodenal pouch after appropriate surgical procedures.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The abdomen is opened through a midline epigastric incision under barbiturate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the common bile duct is isolated. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodeno-jejunosomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of 3 parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch. When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows bile to enter the duodenum in the normal manner. For collection of bile this plug is removed and a long obturator is inserted. The latter effectively isolates the bile secretion from duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured by a further purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula. The cannula is left open to drain blood and secre-

tions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Daily checks of the cannula are advisable to ensure that the plug remains tight. The animals receive normal kennel food and water *ad libitum*.

The dogs are allowed at least 4 weeks to recover. Eighteen hours prior to the experiment food is withdrawn but water allowed *ad libitum*. The long hollow obturator is inserted and bile collected for 15 min periods. After 1 h pretest time, the test compound is given either orally or intravenously.

EVALUATION

Secretion of bile is measured at 15 min intervals and volume and bile contents are determined from 1 ml samples. The values are compared with pretest data. The remaining bile is re-infused into the duodenum via the hollow obturator.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described several techniques for fistulae of the gall bladder and also for the fistula of the ductus choledochus in dogs.

An abdominal incision about 10 cm is made on the median line. The duodenum is pulled out and the orifice of the large (first) pancreatic duct is found. The orifice of the ductus choledochus with the orifice of the small (second) pancreatic duct is situated on the other side of the intestine some 2 or 3 cm nearer the stomach. The ductus choledochus goes straight from the gallbladder to the duodenum; further it lies parallel to it and at its end it is attached to the wall of the duodenum. The small pancreatic duct goes from the gland straight to the duodenum.

At the very beginning of the operation it is useful to cut the ligamentum that goes from the liver to the duodenum, because this facilitates orientation and operating. It is necessary to cut out a piece of the intestinal wall with the orifice of the ductus choledochus. But before this one must prepare off a little bit the intestine from the pancreas so as to be able to close conveniently and securely the hole in the intestine and divide between double ligatures the second pancreatic duct.

On the duodenum around the orifice of the ductus choledochus an incomplete oval figure is now marked with a knife, so that the duct enters this figure through the incomplete part of the oval and has its orifice in the middle of this figure. The length of the oval is about 1.5 cm and its width 1 cm. A suture is then made on the edge of this oval, which is cut out not completely but

leaving a small bridge about 0.5 cm wide between the intestine and the oval; through the bridge the duct enters the oval. The mucosa of this bridge must be completely destroyed with a knife.

The oval piece of the intestine is now turned with the mucosa up and its serosa is sutured to the serosa of the intestine. The hole in the intestine is very carefully closed with two layers of sutures. Two heavy threads are then passed underneath the intestine on either side of the place of operation; they are laid through the abdominal wall and tied after the operation is over. They serve as temporary supporting sutures. The oval piece of the intestine is now sutured with the skin of the abdominal wound and the wound is closed in the usual manner. The supporting sutures must be taken out one day or two days after the operation.

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J.6.1.5

Prevention of Experimental Cholelithiasis

PURPOSE AND RATIONALE

Several animal species develop gallstones, either spontaneously or induced by diet, such as **tupaias** (Schwaier 1979; Schwaier et al. 1979), **ground squirrels** (Fridhandler et al. 1983; MacPherson et al. 1987; MacPherson and Pemsingh 1997), **hamsters** (Holzbach 1984; Cohen et al. 1995) and **owl monkeys** (Pekow et al. 1995).

The **prairie dog** (*Cynomys ludovicianus*) is the animal model that has been used most extensively to study diet-induced cholesterol gallstone formation and the inhibition thereof (Holzbach 1984; Conter et al. 1986; Matoba et al. 1989; Cohen et al. 1990; Broughton et al. 1991; Afdhal et al. 1993; Kam et al. 1996).

Davis et al. (2003) and Saunders et al. (1991) studied the use of statins for the prevention of gallstones in cholesterol-fed prairie dogs.

PROCEDURE

Wild-caught male prairie dogs weighing about 1 kg were kept in quarantine for 3 weeks and fed normal rodent chow. Then the diet was changed to a lithogenic diet consisting of a 1.2% cholesterol formulation for the study period of 28 days. Medication (drug or placebo) was applied through a gastric tube under

slight sedation twice a day. Blood chemistry analysis for total cholesterol and triglycerides was performed on study days 0, 14, and 28.

At the completion of the study period, all animals were placed under general anesthesia and underwent an open cholecystectomy. A subcostal incision was made in the right upper quadrant, and the gallbladder removed using clips on the cystic duct. The gallbladder was then opened and visually inspected for gallstones. Bile from the gall bladder was aspirated for microscopic analysis.

A sample of bile of each animal was examined using light microscopy to determine the presence of cholesterol crystals.

EVALUATION

Statistical analysis was performed using Student's *t*-test for cholesterol and triglyceride data. The Fisher exact test was utilized for analysis of gallstone and cholesterol crystal formation data.

MODIFICATIONS OF THE METHOD

Chapman et al. (1998) established and characterized primary gallbladder epithelial cell cultures in the prairie dog.

Chen et al. (1999) studied biliary sludge and pigmented stone formation in bile duct-ligated guinea pigs.

Stone et al. (1987) developed a rat model to explain the ciclosporin-induced cholestasis observed in transplantation patients.

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J.6.2

Gall Bladder Motility

J.6.2.1

Activity on Isolated Gall-Bladder Strips from Guinea Pigs

PURPOSE AND RATIONALE

Effects on the smooth musculature of the gall bladder can be studied in isolated strips of gall bladder from guinea pigs or cats (Chowdhury et al. 1975; Fara and Erde 1978; Cabrini et al. 1995).

PROCEDURE

Guinea pigs of either sex weighing approximately 400 g are anesthetized with 3 g/kg urethane i.p. The

gall bladder is removed and cut into longitudinal strips 10×3 mm. The strips are suspended in a Krebs solution bath between a stationary hook and an isometric strain gauge. Tension is recorded on a polygraph. The bath is maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2 . After a half-hour stabilization period, test doses of acetylcholine (1 mg/ml) are added to determine viability of the preparation. Dose-response curves for the muscle strips are obtained by introducing one dose of the stimulating agent and waiting until the maximal response to that dose is reached (usually 5–15 min). The bath is then rinsed three times and 15 min allowed before a new dose is tested.

EVALUATION

Concentrations for the maximal response and ED_{50} values are calculated.

MODIFICATIONS OF THE METHOD

Eltze et al. (1997) found that contractions of the isolated guinea-pig gallbladder elicited by muscarinic stimuli are mediated by functional muscarinic M_3 receptors.

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J.6.2.2

Gallbladder Motility in Dogs

PURPOSE AND RATIONALE

Gall bladder motility can be measured in anesthetized dogs with intraluminal manometry.

PROCEDURE

Beagle dogs weighing 15–18 kg are fasted for 16 h prior to the experiment. Median laparotomy is performed under pentobarbital anesthesia (25 mg/kg i.v.). An incision is made in the bile bladder and a 2 mm diameter polyethylene catheter introduced. The catheter is advanced as far as possible to the neck of the gall bladder and tied. The pressure in the interior of the gall

bladder is recorded on a pen recorder via a Statham pressure transducer (P 23 BB) and a frequency measuring bridge.

EVALUATION

Cholecystokinin increases dose-dependent the intraluminal pressure. The preparation can be used for evaluation of cholecystokinin or cholecystokinin-like activity.

MODIFICATIONS OF THE METHOD

Ryan and Cohen (1976) used adult opossums (*Didelphis virginiana*) of either sex, weighing 2.5–3.0 kg.

REFERENCES AND FURTHER READING

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J.6.2.3

Cholecystokinin Activity (Isolated Gallbladder or Intestine)

PURPOSE AND RATIONALE

A sensitive bioassay for cholecystokinin utilizing strips of rabbit gall bladder has been described by Amer and Becvar (1969). Segments of ileum and colon have been used by Paton and Zar (1968) and by Zetler (1984).

PROCEDURE

Male albino rabbits weighing 1.5–2.5 kg are fasted 18 h prior to the experiment. The animals are sacrificed by cervical dislocation and the liver is quickly dissected out. The gallbladder is teased away from the liver and immediately placed in Locke-Ringer solution. The biliary contents are emptied and the gall bladder is cut spirally (right-handed spiral starting from the bile duct) into a strip of muscle tissue 30–40 mm in length and about 5 mm wide. The muscle strip is placed in an organ bath containing Locke-Ringer solution at 37°C continuously bubbled with carbogen. The initial tension of the muscle is adjusted to 0.5 g. The bath fluid is changed every 30 min. The test compound and the standard (range 5–20 mIDU (Ivy dog units) are added alternatively. The maximum of contractions is usually reached after 5 min. Then, the bath fluid is changed. The next dose is applied when the tension has achieved again the starting value.

EVALUATION

The maximum of contraction is taken as endpoint. Using at least two doses of test compound and standard,

parallel line assays can be carried out and potencies ratios can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

This bioassay has the advantage to be sensitive and less time consuming than *in vivo* assays, such as measuring gall bladder motility in the anesthetized dog (see J.6.2.2). However, the assay is not specific for cholecystokinin, since also gastrin and gastrin analogues cause contractions of the isolated bile bladder. This assay allows also to calculate potency ratios of different gastrin analogues.

MODIFICATIONS OF THE METHOD

The whole gallbladder of the mouse or a strip of guinea pig gallbladder is used for evaluation of cholecystokinin-like peptides, ceruletide, ceruletide analogues, and cholecystokinin octapeptide by Zetler (1979, 1984)

Paton and Zar (1968), Zetler (1984), Chang and Lotti (1986), Barthol and Holzer (1987) used isolated segments of ileum and colon from guinea pigs for determination of CCK-like activity.

Makovec et al. (1986), Tachibana et al. (1996) studied the *in vivo* activity of derivatives of CCK in emptying the gallbladder in mice.

Henke et al. (1997) tested CCK-A agonists in the isolated guinea pig gall bladder.

Singh et al. (1995) used gall bladder strips from guinea pigs to evaluate CCK receptor antagonists.

Fukamizu et al. (1998) tested the effect of a cholecystokinin-A receptor antagonist against CCK-8 induced contractions in isolated smooth muscle fibers of gall bladders and longitudinal fibers of ileum from guinea pigs.

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J.6.3

Sphincter Oddi Function

J.6.3.1

Relaxation of Sphincter of Oddi In Vitro

PURPOSE AND RATIONALE

The integrity of the relaxation function the sphincter of Oddi is a prerequisite for normal delivery of bile into the duodenum. Sphincter of Oddi relaxation is mainly executed by non-adrenergic, non-cholinergic (NANC) nerves that are essentially nitrenergic in several species including guinea pigs (Pauletzki et al. 1993) and rabbits (Lonovics et al. 1994).

PROCEDURE

Biliary sphincter of Oddi muscle rings of approximately 6 mm length from adult male New Zealand white rabbits weighing from 3500–4000 g pretreated with various drugs or diet are prepared. The papilla Vater is eliminated and the ampullary part of the muscle rings of approximately 3 mm length are mounted horizontally on two small L-shaped glass hooks one of which is connected to a force transducer attached to a polygraph for measurement and recording of isometric tension. The experiments are carried out in an organ bath (5 ml) containing Krebs bicarbonate buffer which is maintained at 37°C and aerated continuously with carbogen. The initial tension is set at 10 mN and the rings are allowed to equilibrate over

1 h during which period the sphincters develop characteristic 14–19 per min rhythmic contractions. Atropine (1 μM) and guanethidine (4 μM) are continuously present (NANC solution). Changes in isometric tension in response to two consecutive trains of impulses of electrical field stimulation (40 stimuli, 50 V, 0.1 ms and 20 Hz) are then studied.

EVALUATION

The data representing changes in isometric tension expressed as means ± standard deviation are evaluated by means of analysis of variance (ANOVA) followed by a modified Student's *t*-test for multiple comparisons according to Bonferroni's method. Changes are considered statistically significant at P-values smaller than 0.05.

MODIFICATIONS OF THE METHOD

In addition to studies with the isolated sphincter of Oddi of rabbits (Slivka et al. 1994; Sari et al. 1998; Jia and Stamler 1999) and guinea pigs (Harrington et al. 1992; Gocer et al. 1995; Lu et al. 1997), several pharmacological studies were reported with the isolated sphincter of Oddi of **opossum** (Perodi et al. 1990; Allescher et al. 1993), and of the **Australian brush-tailed possum** (*Trichosurus vulpecula*) (Baker et al. 1992, 1996).

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J.6.3.2

Function of Sphincter of Oddi In Vivo

PURPOSE AND RATIONALE

Dogs were used for *in vivo* studies of the function of the sphincter of Oddi (Sarles 1986; Pozo et al. 1990; Matsumara et al. 1991; Kobayashi et al. 1994; Wang et al. 1998). Shima et al. (1998) recorded the spontaneous motility and the response to cerulein on the canine sphincter of Oddi using a constant-perfusion technique.

PROCEDURE

Mongrel dogs weighing 7–9 kg fasted overnight are anesthetized with 25 mg/kg intravenous pentobarbital sodium and are maintained under adequate anesthesia with 12.5 mg/kg intravenous pentobarbital sodium as required. Systemic blood pressure is monitored through a catheter placed into the femoral artery. A femoral vein is cannulated and used for systemic administration of Ringer's solution and drugs.

After an upper median laparotomy, a small longitudinal incision is made in the common bile duct. Two catheters (outer diameter 2.0 mm) are cannulated and tied in the bile duct to avoid any leaks and the occlusion of the orifice of the catheters. One is distally placed at 5 mm proximal to the choledochoduodenal junction and is used to perfuse the sphincter of Oddi with saline at a rate of 0.12 ml/min using an infusion pump. The other is proximally placed and used to siphon off the hepatic bile. Pressure changes are recorded on a polygraph through a pressure transducer which is placed between the infusion pump and the catheter.

EVALUATION

To evaluate the effects of intravenously administered drugs on the sphincter of Oddi, a motility index is calculated by measuring the square between the zero line and the trace of the sphincter of Oddi pressure changes per minute.

Results are expressed as means \pm SEM; *n* is the number of independent observations of different animals. The paired and unpaired *t*-tests are used for statistical analysis. *P* < 0.05 is considered significant.

MODIFICATIONS OF THE METHOD

Thune et al. (1992, 1995) studied simultaneously the flow resistances in the common bile duct and main pancreatic duct sphincters in anesthetized **cats** using a perfusion technique.

Elbrønd et al. (1994) prepared the sphincter of Oddi and duodenum in anesthetized **rabbits** with perfused catheters and bipolar electrodes. Increasing, successive doses of cholecystokinin were administered intravenously every 15th min. The digitized recordings were scored on a computer in control and stimulatory cholecystokinin sequences.

Further studies in **rabbits** were performed by Nakamura (1996), Chiu et al. (1998).

Opossums were used by Calabuig et al. (1990), Hanyu et al. (1990), Cullen et al. (1996), Herrmann et al. (1999) to study the function of the sphincter of Oddi.

Several authors used the **Australian brush-tailed possum** (*Trichosurus vulpecula*) for studies of the function of the sphincter of Oddi *in vivo* (Baker et al. 1990; Saccone et al. 1992; Cox et al. 1998a, b; Huang et al. 1998).

The **prairie dog** was used by several authors to study the function of the sphincter of Oddi (Ahrendt et al. 1992; Kaufman et al. 1993; Thierney et al. 1994).

Pasricha et al. (1995) reported a model in **pigs** for endoscopic biliary manometry, similar in technique to the procedure in humans.

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J.7 Pancreatic Function

J.7.0.1 Acute Pancreatic Fistula in Rats

PURPOSE AND RATIONALE

The effect of exogenous hormones, e. g. secretin, or other drugs on pancreas secretion can be measured in rats with acute pancreas fistula.

PROCEDURE

Male Sprague Dawley rats weighing 150–200 g are used. Eighteen hours prior to the experiment food is withdrawn with free access to water. Groups of 4–5 rats are used for each dose of drug evaluation, the control group consists of at least 7 rats. The animals are anesthetized with 5 ml 25% urethane solution i.m. and the trachea cannulated. The abdomen is opened by a mid-line incision and the pylorus ligated. The proximal part of the bile duct is ligated near the hepatic porta. The bile is drained via a thin polyethylene tube into the duodenum. The distal part of the bile duct with the orifices of pancreatic ducts is cannulated with another thin polyethylene tube. The pancreatic juice is collected in graduated microvessels. After a pre-test period of 60 min, the test compounds are applied intravenously or intraduodenally.

EVALUATION

The secretion after injection of the test compound is compared with the pre-test values. Secretin increases pancreatic secretion volume in a dose-dependent manner. Activity ratios for unknown preparations can be calculated by 2+2 assays in comparison with the international standard.

MODIFICATIONS OF THE METHOD

Guan et al. (1990) inserted two separate cannulae for bile and pancreatic juice to Wistar rats under methoxyfluorane anesthesia. Both fluids were returned to the intestine. Placing the rats in modified Bollman-

type restraint cages, experiments could be performed after a few days in conscious animals.

Ito et al. (1994) studied the inhibition of CCK-8-induced pancreatic amylase secretion by a cholecystokinin type-A receptor antagonist in rats.

Niederau et al. (1989) compared the effects of CCK receptor antagonists on rat pancreatic secretion *in vivo*. Output of amylase in pancreatico-biliary secretion was measured after various doses of caerulein. The effects of high caerulein doses were dose-dependent inhibited by CCK-antagonists.

Alvarez and Lopez (1989) studied the effect of alloxan diabetes on exocrine pancreatic secretion in the anesthetized **rabbit**. After a 14–15 h fasting period, but with free access to water, rabbits weighing about 2.0 kg are anesthetized by intravenous injection of 1.0 g/kg urethane. After tracheotomy, a median laparotomy is performed, the main pancreatic duct is exposed and cannulated near its entrance to the duodenum following ligation of the pylorus and cannulation of the bile duct for deviation of bile to the exterior.

Kim et al. (1993) studied the effect of [(CH₂NH)4,5]secretin on pancreatic exocrine secretion in **guinea pigs** and rats using an acute pancreatic fistula preparation.

Niederau et al. (1990), Tachibana et al. (1996) determined pancreatic exocrine secretion in **mice**. Because the cannulation of mouse pancreatic duct is not possible for technical reasons, the amount of amylase was determined *in vivo*. Five min after i.v. administration of test drugs, mice were sacrificed and a 5 cm-duodenal loop was removed. The duodenal contents were washed out with 1.0 ml ice-cold saline and collected for amylase activity.

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J.7.0.2

Exocrine Secretion of Isolated Pancreas

PURPOSE AND RATIONALE

Procedures for isolation and perfusion of rat pancreas in order to record membrane potential and effective membrane resistance during the collection of perfusates and for simultaneous measuring of flow of pancreatic juice were described by Kanno (1972), Kanno and Saito (1976), Kanno et al. (1976), Saito et al. (1980). Similar techniques to measure exocrine secretion of the isolated pancreas have been used by other groups (Penhos et al. 1969; Mann and Norman 1984; Trimble et al. 1985; Norman and Mann 1986, 1987; Norman et al. 1989; Park et al. 1993).

PROCEDURE

Male Sprague Dawley rats weighing about 250 g are fasted for 24 h before the experiments but are allowed to drink water. Under ether anesthesia and after cannulation, the vascular system and the common duct are separately perfused. The rectum and the transverse colon are separated from the pancreas following ligation and sectioning of their vascular supply. Perfusion is performed through the hepatic end of the common duct with the outlet at the duodenal end. The **flushing preparation** is used when continuously recording the transmembrane potential and, in this preparation, the common duct is flushed at a constant pressure of 5 cm H₂O following cannulation of both ends with stainless steel tubes. The **draining preparation** is used during the electrical measurements are made from as many acinar cells as possible in every 10 min. In the draining preparation, the hepatic end of the duct is ligated and the pancreatic juice collected from the duodenal end following cannulation with a stainless steel tube. In both preparations, the inlets of the vascular perfusion are the superior mesenteric artery and the coeliac artery, and the outlet is the portal vein. The rate of vas-

cular flow is kept constant at 1 ml/min with the aid of a roller pump. The animal is then killed by cutting the carotid arteries and the perfusion begins. The spleen is removed after section of its vascular supply close to the hilum, care being taken to avoid interference with the supply from the splenic artery. The blood supplying stomach and liver is stopped by tying the hepatic artery and gastric arteries. The superior mesenteric vein and the descending branch of the superior mesenteric artery are then ligated. The mesentery with its embedded whole pancreas and the attached duodenum is then removed and mounted on a paraffin block in a lucite chamber containing 20 ml of standard Krebs-Henseleit solution. The contents of the duodenum is then drained with a polyethylene tube. The level of the standard Krebs-Henseleit solution in the bath is kept constant with a siphon. The temperature of the preparation and perfusing Krebs-Henseleit solution containing 8 vol% erythrocytes is maintained at 37°C. The perfusion solution in the reservoir is continuously bubbled with 5% CO₂ in O₂.

The rate of flow of pancreatic juice is measured by attaching a calibrated tube made of silicone-rubber to the free end of the pancreatic duct cannula. Every 10th min the tube is replaced and the rate of flow of pancreatic juice is noted. The sample is diluted with Krebs-Henseleit solution up to 100 ml and the amount of amylase assessed.

Intracellular recordings are made from the pancreatic acinar cells by manually advancing KCl-filled microelectrodes under direct visual control. The microelectrode is connected to the probe of a solid state pre-amplifier. Resting potentials are observed at the screen of a cathode ray oscilloscope and simultaneously recorded on a direct visual oscillograph.

Measurements are made every 10 min for half an hour as base level, followed by measurements every 10 min during infusion of drug for one hour.

EVALUATION

All results are expressed as mean \pm SEM. Statistical analysis is performed using Student's *t*-test.

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J.7.0.3

Chronic Pancreatic Fistula in Rats

PURPOSE AND RATIONALE

Suguiyama et al. (1996) described the preparation of a chronic pancreatic fistula in the WBN/Kob rat, a strain which develops spontaneously chronic pancreatitis (Ohashi et al. 1990; see also J.7.0.14).

PROCEDURE

Male WBN/Kob rats at an age of 2 months are used. Before surgery, the rats have free access to chow and water. After an 18-h fast, laparotomy is performed through a midline incision under general anesthesia with ether. A polyethylene catheter (inside diameter 0.28 mm; outside diameter 0.61 mm, length 30 cm), as an external pancreatic fistula, is inserted into the common biliary pancreatic duct immediately proximal to the ampulla of Vater. The bile duct is ligated proximally to the pancreas. Another catheter is introduced into the bile duct distal to the confluence of the hepatic ducts, and the opposite end of the catheter is inserted into the duodenum near the ampulla through the stomach to drain the bile into the intestine. A third catheter is inserted in the duodenum through the stomach to return pancreatic juice. A fourth catheter is placed in the femoral vein, and 0.15 M sodium chloride is continuously administered at a flow rate of 1 ml/h with a sy-

ringe pump. All catheters are brought into the abdominal cavity or the femoral region through a subcutaneous tunnel starting in the middle portion of the tail. The rat is then placed prone in a cage with the proximal portion of the tail fixed to a side wall of the cage. When awakened, the rat is allowed access to food and water ad libitum. Pancreatic juice is collected and continually re-circulated with a syringe pump every 6 h until the secretion test.

The secretion test is started 3 days after surgery. After a 12-h fast, pancreatic juice is collected every 30 min in a plastic syringe. The juice volume is determined by weighing. A 50- μ l sample is taken for the analysis of juice composition, and the remaining juice is returned to the duodenum for the next 30-min collection period.

After a 30-min basal period, the test compound is administered in graded doses, each dose for 30 min.

The bicarbonate concentration of pancreatic juice is measured with a microgasometer (Natelson 1958) in a sample size of 10 μ l. Protein concentration is determined by measuring optical density at 280 nm with purified bovine trypsinogen as standard.

EVALUATION

All values are presented as mean \pm SEM. Results are analyzed by means of the Wilcoxon test. Differences are considered significant at $p < 0.05$.

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J.7.0.4

Acute Pancreatic Fistula in Dogs

PURPOSE AND RATIONALE

The effect of exogenous hormones, e. g. secretin or gastrin, or of vagal stimulation, on pancreatic secretion can be measured in dogs with acute pancreas fistulas.

PROCEDURE

Beagle dogs of either sex weighing 12–20 kg are used. The animals are fasted for a 24-h period and then anes-

thetized with 25 mg/kg sodium pentobarbital i.v. After opening the abdomen along the mid-line, the pyloric sphincter is ligated and the common bile duct cannulated to prevent the entry of acid chyme and bile into the duodenum. The bile is allowed to drain. The pancreas is gently exposed and the major pancreatic duct ligated. A polyethylene tube of 2 mm diameter is inserted into the minor pancreatic duct for collection of the secretion. The left femoral vein is cannulated for continuous infusion or i.v. injection. The pancreatic juice is collected in an ice-bath in a special tapered tube with fine calibrations for measuring volumes of less than 1 ml.

At the end of each collection period, the volume is recorded, and the bicarbonate content determined titrimetrically. Furthermore, pancreatic enzymes, such as amylase, are determined in the samples. Determination of protein concentrations in the pancreatic juice can be used as end-point since the total protein concentration is proportional to the individual enzymes (Keller et al. 1958). In a pretest period of 10 min, samples are collected every 2 min. Then, the test compound is injected intravenously and the pancreatic juice is collected every 2 min.

EVALUATION

The secretion after injection of the test compound is compared with the pre-test values. Secretin increases pancreatic volume and bicarbonate secretion in a dose-dependent manner. Activity ratios for unknown preparations can be calculated by 2+2 points assays in comparison with the international standard.

Moreover, the model can be used for standardization of gastrin analogues.

MODIFICATIONS OF THE METHOD

Glad et al. (1996) tested the influence of gastrin-releasing peptide on acid-induced secretin release and pancreatobiliary and duodenal bicarbonate secretion in Danish country strain **pigs** weighing between 22 and 30 kg. The animals, starved overnight with free access to water, were premedicated with 4 mg/kg i.m. azaperone, and with 5 mg/kg i.p. metomidate. After 20 min a cannula was placed in an ear vein, and 5–10 mg/kg metomidate was given i.v. followed by intubation and artificial respiration with 50% O₂ and 50% N₂O. Anesthesia was maintained with an intravenous bolus infusion of 0.53% α -chloralose.

Both external jugular veins were cannulated for infusion of saline or drugs. A femoral artery was cannulated for withdrawal of blood samples and recording of blood pressure. After laparotomy the cystic duct

was ligated, and the common hepatic duct and the pancreatic duct were catheterized. The duodenal segment was defined as extending from the pylorus to the ligament of Treitz. A Foley catheter was passed through the pylorus into the proximal part of the duodenum and inflated. Distal to the pylorus the pancreaticoduodenal arteries, veins and nerves were dissected, and a double ligature was passed under these structures and tied around the duodenum. At the ligament of Treitz an inflated Foley catheter was placed in the distal part of the duodenum and tied with a suture around the duodenum. A catheter was placed through a splenic branch of the left gastroepiploic vein and advanced through the lienal vein to the portal vein.

The flow of pancreatic juice and bile was tested before and after the experiment by means of an intravenous bolus of 5 pmol/kg secretin. Before the experiment the duodenum was continuously perfused at a rate of 2 ml/min for 435 min with isotonic saline containing phenol red (10 mg/l) as a marker. After drug treatment (intravenous infusion of gastrin-releasing peptide or duodenal HCl perfusion) pancreatic and hepatic secretions were collected in 15-min periods and the volumes determined by weighing. Duodenal effluents were collected in 15-min periods and phenol red concentrations determined spectrophotometrically. Blood sampled were withdrawn for determination of secretin by radioimmunoassay.

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J.7.0.5

Chronic Pancreatic Fistula in Dogs

PURPOSE AND RATIONALE

Herrera et al. (1968) described a special cannula which can be used to obtain pancreatic juice or bile from a duodenal pouch after appropriate surgical procedures (Preshaw and Grossman 1965).

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The abdomen is opened through a midline epigastric incision under barbiturate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the main pancreatic duct is isolated. The proximal level of the duodenal section lies immediately distal to the opening of the common bile duct and the distal level of section lies 2.5 cm distal to the main pancreatic duct. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodeno-jejunostomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of 3 parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch.

When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows pancreatic juice to enter the duodenum in the normal manner. For collection of juice this plug is removed and a long obturator is inserted. The latter effectively isolates the pancreatic secretion from other duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured further by a purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula.

The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Checking the cannula daily is advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 2 weeks to recover. Eighteen hours prior to the experiment food is with-

drawn but water allowed ad libitum. The long obturator is inserted and pancreatic juice collected for 15 min periods. After 1 h pretest time, the test compound is given either orally or intravenously.

EVALUATION

Secretion of pancreas juice is measured at 15 min intervals and volume and enzyme content determined. The values are compared with pretest data.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described details of the technique as recommended by Pavlov (1902) as well as his own modification.

Konturek et al. (1976, 1984) performed experiments with chronic gastric fistulas in **cats** as well as in **dogs** to compare the species-specific activities of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion.

Ninomiya et al. (1998) studied the effects of a cholecystokinin A receptor antagonist on pancreatic exocrine secretion stimulated by exogenously administered CCK-8 in conscious dogs with chronic pancreatic fistula.

Garvin et al. (1993) described distal pancreatectomy with autotransplantation and pancreaticocystostomy in dogs.

Kuruda et al. (1995) developed a new technique in dogs for pancreatigo-gastrointestinal anastomosis that consists of pancreatectomy using the ultrasonic dissector and implantation of the pancreatic duct into the gastrointestinal tract without suturing the pancreatic parenchyma.

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J.7.0.6

Somatostatin Activity

PURPOSE AND RATIONALE

Somatostatin is a neuropeptide, also called somatotrophin release inhibiting factor (SRIF or SRIF-14), which occurs not only in the brain, but also in a variety of peripheral tissues. Two other related peptides somatostatin-28 (SRIF-28) and cortistatin (Vasilaki et al. 1999), also occur naturally (Humphrey 1998). Somatostatin acts via G-protein coupled receptors of which five subtypes are cloned (Hoyer 1998).

Somatostatin analogues and antagonists have been tried for a variety of indications (Bass et al. 1996; Coy and Taylor 1996; Papageorgiou and Borer 1996; Rohrer et al. 1998; Yang et al. 1998; Hocart et al. 1999). The long-acting octapeptide somatostatin analogue, octreotide, has been studied thoroughly (Ambler et al. 1996; Danesi and Del Tacca 1996; Hoffmann et al. 1996; Paran et al. 1996) and gained clinical use as gastric antisecretory agent.

Somatostatin inhibits basal pancreatic secretion as well as secretion stimulated by food, cholecystokinin, and secretin. Inhibition of pancreatic secretion can be used as bioassay to compare synthetic analogues with the original somatostatin.

PROCEDURE

Male Wistar rats weighing 300–400 g are anesthetized with methoxyfluorane and prepared with silastic cannulae. Two cannulae drain pancreatic juice and bile separately, one cannula is inserted into the duodenum for return of bile and pancreatic juice to the intestine and for intestinal infusion. Another cannula is located in the abdominal cavity and a fifth cannula into the right jugular vein for drug infusion. After surgery, the rats are placed into modified Bollman-type restrain cages. The rats have free access to food and water. Dur-

ing recovery and between the experiments, pancreatic juice and bile are collected and continuously returned to the intestine by a servo-system consisting of a collection tube in a liquid-level photodetector coupled to a peristaltic pump.

Experiments are performed on the third to eighth days postoperatively. The rats are assigned to treatment groups on a random basis and, treatment days are equally divided among the third to eighth postoperative days. The rats are fasted overnight before each experiment. Bile and pancreatic juice are collected separately every 30 min; the volume of pancreatic juice is measured, and 10 ml is taken for protein assay. Different doses of the test drug and the standard are given as continuous i.v. infusion over a period of 2 h.

EVALUATION

Inhibition of pancreatic fluid and protein output is calculated compared to control infusion and expressed as percentage. From dose-response curves activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Konturek et al. (1985) and Susini et al. (1980) studied the effect of somatostatin analogues on pancreas secretion in dogs.

Cai et al. (1986) reported on the biological activity of octapeptide analogs of somatostatin assaying GH concentrations in blood samples of anesthetized rats by RIA and studying the inhibition of gastric secretion on dogs.

Biological actions of prosomatostatin have been described by Meyers et al. (1980) using *in vitro* and *in vivo* GH bioassays.

Taylor et al. (1996) employed the technique of cytosensor microphysiometry for real-time evaluation of somatostatin subtype receptor activity in CHO-K1 cells stably expressing the human sst₂ receptors.

Cytotoxic analogs of somatostatin have been synthesized and evaluated (Nagy et al. 1998).

Gilon et al. (1998) studied a synthetic receptor 5-selective somatostatin analogue *in vivo* in rats. The compound inhibited bombesin- and caerulein-induced amylase and lipase release from the pancreas without inhibiting growth hormone or glucagon release.

Jeandel et al. (1998) described the effects of two somatostatin variants on receptor binding, adenylyl cyclase activity and growth hormone release from the frog pituitary.

Hofland et al. (1994) determined relative potencies of somatostatin analogs on the inhibition of growth

hormone release by cultured human endocrine tumor cells and normal rat anterior pituitary cells.

Rohrer et al. (1998) and Rohrer and Schaeffer (2000) described rapid identification of subtype-selective agonists of the somatostatin receptor through combinatorial chemistry.

Hoyer et al. (2004) characterized *in vitro* a non-peptide somatostatin sst₁ receptor antagonist.

Reubi et al. (2000) described SST₃-selective potent peptidic somatostatin receptor antagonists.

Nunn et al. (2003a, 2003b) reported properties of putative small-molecule somatostatin sst₂ receptor-selective antagonists.

Using high-throughput parallel synthesis, Moinet (2001) prepared non-peptide ligands for the somatostatin sst₃ receptor.

Gademann et al. (2001) reported that peptide folding induces high and selective affinity of a linear and small β -peptide to the human somatostatin receptor 4.

Nunn (2003c) described β^2/β^3 -di- and α/β^3 -tetrapeptide derivatives as potent agonists at somatostatin sst₄ receptors.

Somatostatin analogs labeled with ^{99m}technetium, ¹¹¹indium, and ⁹⁰yttrium were developed for tumor diagnosis and therapy (O'Byrne and Carney 1996; Pearson et al. 1996; Stolz et al. 1996, 1998; Thakur et al. 1996).

Radioimmunoassays for somatostatin have been developed (Arimura et al. 1975; Gerich et al. 1979; Patel and Reichlin 1979; Patel 1984) and are available as commercial kits.

Siehler et al. (1998) reported that [¹²⁵I]Tyr¹⁰-cortistatin₁₄ labels all five somatostatin receptors.

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J.7.0.7

Receptor Binding for Somatostatin

GENERAL CONSIDERATIONS

A family of five somatostatin receptor subtypes from various species including man has been described (Yamada et al. 1992; Reisine and Bell 1995; Hoyer et al. 1995; Bruns et al. 1996; Liapakis et al. 1996; Patel and Srikant 1994; Hoyer et al. 1995; Patel et al. 1996; Nilsson and Folkesson 1997; Patel 1997; Pscherer et al. 1996; Shimon et al. 1997; Humphrey et al. 1998; Moller et al. 2003; Olias et al. 2004). There are several endogenous ligands, such as somatostatin-14, somatostatin-28 and the corticostatins (Meyerhoff et al. 1992; De Lecca 1996; Fukusumo 1997). A number of synthetic peptide analogues have been developed and their relative affinities at human recombinant receptors in radioligand receptor studies have been established (Raynor et al. 1992; Bass et al. 1996; Coy and Taylor 1996; Pearson et al. 1996; Piwko et al. 1996, 1997a, b).

Greenman and Melmed (1994) evaluated the expression of three somatostatin receptor subtypes (SSTR3, SSTR4, and SSTR5) in pituitary tumor specimens. SSTR3 expression was studied by reverse transcription coupled to polymerase chain reaction, whereas SSTR4 and SSTR5 expression was determined by ribonuclease protection assay.

Somatostatin occurs not only in the hypothalamus and in the gut, but also in several other organs. Somatostatin₁ receptors in the nucleus accumbens have been found to mediate the stimulatory effect of somatostatin on locomotor activity in rats (Raynor et al. 1993a). Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28 has been described by O'Carroll et al. (1992).

Cloning and expression of a mouse somatostatin receptor (SSTR 2B) has been reported by Vanetti et al. (1992). Pharmacological properties of two cloned somatostatin receptors have been described by Rens-Domiano et al. (1992).

Subtype-selective peptides have been identified by Raynor et al. (1993b). The somatostatin receptor, SSTR3, coupled to adenylyl cyclase, has been cloned by Yasuda et al. (1992). Somatostatin receptors SSTR4 and SSTR5 have been cloned and characterized by Raynor et al. (1993c).

The somatostatin receptor SST₁ mediates inhibition of central neurons (Viollet et al. 1997).

The somatostatin receptor SST₂ inhibits growth hormone release (Raynor et al. 1993b), inhibits parietal cell function and ion secretion in rat distal colon (McKeen et al. 1996; Warhurst et al. 1996; Wyatt et al. 1996), and inhibits neurons in the rat locus coeruleus (Chessell et al. 1996). High basal gastric acid secretion has been found in somatostatin receptor subtype 2 knockout mice (Martinez et al. 1998). The cytoplasmic tail of the somatostatin receptor SST₂ undergoes alternative splicing giving rise to two isoforms, SST_{2A} and SST_{2B} (Schulz et al. 1998).

The somatostatin receptor sst_3 mediates relaxation of isolated gastric smooth muscle cells (Gu et al. 1995).

The fourth human somatostatin receptor has been cloned and characterized by Rohrer et al. (1993).

The somatostatin receptor sst_5 may have a role in inhibition of hormone release from the pituitary (Tallent et al. 1996) and pancreas (Rossowski and Coy (1994) and mediates the antiproliferative effect of somatostatin in vascular smooth muscle (Lauder et al. 1997).

PROCEDURE

Crude membrane preparations are obtained by homogenizing (Polytron, setting 6, 15 s) tumor cell cultures (e. g. human SCLC line NCI-H69, rat pancreatic tumor AR42J) or rat tissues (lung, pancreas, cerebral cortex) in ice-cold 50 mM Tris-HCl and centrifuging twice at 39,000 g (10 min), with an intermediate resuspen-

sion in fresh buffer. The final pellets are resuspended in 10 mM Tris-HCl for assay. Aliquots of the membrane preparation are incubated for 25 min at 30°C with [¹²⁵I-Tyr¹¹]SRIF (2000 Ci/mmol, Amersham) in 50 mM HEPES (pH 7.4) containing bovine serum albumin (10 mg/ml; fraction V, Sigma), MgCl₂ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 mg/ml), and phenyl-methyl-sulfonyl fluoride (0.02 mg/ml). The final assay volume is 0.3 ml. The incubations are terminated by rapid filtration through Whatman GF/C filters (pre-soaked in 0.3% polyethylenimine) under reduced pressure. Each tube and filter is then washed three times with 5 ml aliquots of ice-cold buffer. Specific binding is defined as the total [¹²⁵I-Tyr¹¹]SRIF bound minus that bound in the presence of 200 nM unlabeled SRIF.

EVALUATION

The binding parameters are calculated from the experimental data by non-linear least-squares regression analysis using an appropriate computer program.

CRITICAL ASSESSMENT OF THE METHOD

Somatostatin occurs in many organs and has more activities than anticipated at its discovery. Therefore, the occurrence of a family of receptors rather than a single receptor is not surprising.

MODIFICATIONS OF THE METHOD

Receptor scintigraphy with a radioiodinated somatostatin analogue has been described by Bakker et al. (1990).

The tissue-selective binding of somatostatin-14 and somatostatin-28 in rat brain was studied by Srikant et al. (1990).

Feniuk et al. (1993) characterized somatostatin receptors in guinea-pig isolated ileum, vas deferens and right atrium.

Yang et al. (1998) reported synthesis and biological activities of potent peptidomimetics selective for somatostatin receptor subtype 2 by testing inhibition of forskolin-stimulated cAMP accumulation in a mouse cell line that contains a cAMP response element fused to the *E. coli* β-galactosidase gene and by measuring growth hormone release from primary cultures of rat anterior pituitary cells.

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J.7.0.8

Secretin Activity

PURPOSE AND RATIONALE

Synthetic derivatives of secretin can be tested for activity against the standard using the bioassay of a chronic pancreatic fistula in dogs. Moreover, stability of secretin preparations has to be checked when using this bioassay.

PROCEDURE

Dogs with a chronic pancreatic fistula prepared according to Preshaw and Grossman (1965), Herrera et al. (1968) (see J.6.1.4) are used. Eighteen hours prior to the experiment food is withdrawn but water allowed ad libitum. Pancreatic juice is collected for 15 min periods. After 1 h pretest time, a test dose of 0.2 KE/kg Karolinska-secretin is given intravenously. In one hour intervals the test preparation or the standard is given alternatively in logarithmic doses between 0.1 and 1.6 KE/kg intravenously. The volume of pancreas juice secreted during 30 min after injection is used as parameter.

EVALUATION

Using at least two doses of test compound and standard, parallel line assays can be carried out and potencies can be calculated. Each of 4 animals receives two doses test compound and two doses standard, the application scheduled according to a Latin square. Potency ratios with confidence limits are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method is time consuming and needs dogs with chronic fistula. Nevertheless, for final evaluation of a test compound this bioassay can not be avoided.

MODIFICATIONS OF THE METHOD

The standardization of secretin has been described as early as 1952 by Burn et al. using anesthetized *cats*. After laparotomy the pylorus and first duodenal loop are brought outside and turned to the animal's left side. The bile duct is identified by gently squeezing the bile-bladder. The pancreatic duct usually enters the duodenum about 2 mm below the entry of the bile-duct.

Starting about 2 cm below the bile duct, the pancreas is separated from the duodenum by blunt dissection, the blood vessels being tied and divided where necessary. When the pancreatic duct is reached, two silk ligatures are placed around and a length of 5 mm is dissected. A dose of secretin is injected intravenously and one of the ligatures is then tied as near the duodenum as possible. The duct is then cut and a flow of juice is usually visible. A small cannula is tied into the duct and attached to a rubber tube and L-piece whose position is adjusted so that the flow of juice continues. The cannula and the L-piece are clamped in position and the abdomen is closed.

The relation between the dose injected and the number of drops of pancreatic juice secreted is almost linear, but the slope differs in different cats. In making a comparison between two preparations, the dose-response relation is first determined for the standard preparation by administration of three or more different doses. A dose of the test preparation is then injected and the effect compared with the curve of the standard. Further doses of the test preparation and comparison of the dose-response curves allow the calculation of the relative potency.

Izzo et al. (1989) studied the internalization of labelled secretin into isolated pancreatic acinar cells of *rats*.

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J.7.0.9

Receptor Binding for Secretin

PURPOSE AND RATIONALE

Secretin receptor antagonists, such as reduced peptide bond pseudopeptide analogues of secretin, inhibit the binding of ¹²⁵I-secretin to secretin receptors in pancreatic acini (Haffar et al. 1991).

Radioimmunoassays for secretin have been developed (Boden and Wilson 1979; Chang and Chey 1980) and are available as commercial kits.

PROCEDURE

Dispersed acini from guinea pig pancreas are prepared according to Peikin et al. (1978) and Jensen et al. (1982). After sacrifice, the pancreas of a guinea pig is immediately removed and trimmed of fat and mesentery. A suspension of dispersed acini is prepared by injecting the pancreas 5 times with 5 ml of digestion solution composed of standard incubation solution plus purified collagenase (0.12 mg/ml). The pancreas is incubated for 4 sequential 10-min periods at 37°C in a Dubnoff incubator at 160 oscillations per min. The gas phase is 100% O₂. The digestion solution is discarded, and the tissue is washed 3 times with 5 ml of standard incubation solution. The tissue is dispersed by passing it 5 times through each of a series of siliconized glass pipettes of decreasing bore (3, 1, and 0.5 mm). Large fragments and the duct system are discarded. The suspension of dispersed acini is layered over standard incubation solution containing 4% albumin and centrifuged at 800 g. The supernatant is discarded, and the acini are washed twice with standard incubation solution containing 4% albumin.

¹²⁵I-Secretin and ¹²⁵I-labeled analogues are prepared using chloramine T and a method described by Chang and Chey (1980), Jensen et al. (1983), Zhou et al. (1989). They are separated from ¹²⁵I using a disposable C₁₈ cartridge (Sep-Pak) and then separated from unlabeled peptide with reverse phase HPLC using a 4.6-mm × 25-cm column of mBondapak C₁₈. The column is eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 0 to 54% acetonitrile in 60 min using a flow rate of 1.0 ml/min.

For binding of ¹²⁵I-secretin incubations are performed containing 0.25 ml of cell suspension of pancreatic acini (one pancreas in 10 ml of standard incubation buffer) and 50 pM ¹²⁵I-secretin with or without 1 mM secretin. Nonsaturable binding of ¹²⁵I-secretin is measured as the amount of radioactivity associated with acini when the incubation contains 50 pM ¹²⁵I-secretin plus 1 mM secretin. Secretin antagonists are added in various concentrations. Saturable binding of ¹²⁵I-secretin is expressed as percentage of radioactivity bound in the absence of the antagonists.

EVALUATION

K_i values are calculated from percent of controls of bound ¹²⁵I-secretin using the equation:

$$K_i = [R/(R - 1)][S B/(S + A)]$$

where R = the observed saturable binding of ¹²⁵I-secretin in the presence of the antagonist (B) expressed

as a fraction of that obtained when B is not present. A is the concentration of ¹²⁵I-secretin (0.05 nM), S is the k_d determined by Scatchard analysis.

MODIFICATIONS OF THE METHOD

Bawab et al. (1991) characterized the down regulation of the ¹²⁵I-secretin binding sites and the associated desensitization of the secretion receptor-cAMP system in rat gastric glands.

Molecular cloning and expression of a cDNA encoding the secretin receptor in COS cells was reported by Ishihara et al. (1991).

Steiner et al. (1993) localized secretin receptors mediating rat stomach relaxation by autoradiography of frozen sections of the rat stomach with ¹²⁵I-labeled porcine secretin.

Ulrich et al. (1993) studied the intrinsic photoaffinity labeling of native and recombinant rat pancreatic secretin receptors.

Vilardaga et al. (1994) investigated the properties and regulation of the coupling to adenylate cyclase of secretin receptors stably transfected in Chinese hamster ovary cells.

Molecular cloning, expression and functional characterization of a human secretin receptor was reported by Chow (1995), Patel et al. (1995), of the rabbit secretin receptor by Svoboda et al. (1996). The full-length human secretin receptor cDNA was subcloned into the mammalian expression vector pRc/CMV and expressed in cultured CHO cells (Ng et al. 1999). Intracellular cAMP accumulation of the stably transfected cells was measured by a radioimmunoassay, while the extracellular acidification rate was measured by the Cytosensor microphysiometer.

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- Isolated pancreatic acini of rats can be used for evaluation of cholecystokinin activity of synthetic derivatives and for plasma CCK-like activity (Liddle et al. 1984; Höcker et al. 1990; Schmidt et al. 1991).

PROCEDURE

For plasma determinations, CCK or inhibitors are extracted from a plasma sample using PR-18 cartridges (Merck, Darmstadt, Germany). One ml plasma is diluted with 4 ml 0.1% trifluoroacetic acid and applied to a cartridge. After a wash (15 ml), CCK and inhibitor are eluted with acetonitrile/water (80:20, v/v) and lyophilized. Pancreatic acini are prepared from female Sprague-Dawley rats weighing 180–200 g, 1 to 2 weeks post ovariectomy, by enzymatic digestion of pancreas with collagenase (Jensen et al. 1982). Test compounds, or extracted material reconstituted with Krebs-Ringer HEPES buffer, or CCK standard is incubated with acini (final volume 0.25 ml). Lipase is measured with an autoanalyser (e. g., Hitachi type 705 or 805). Release is calculated as percent of the initial content determined in each incubation vial.

EVALUATION

CCK bioactivity is determined by comparison with a CCK-8 standard curve. Results are expressed as CCK-like bioactivity.

MODIFICATIONS OF THE METHOD

Amblard et al. (1998) evaluated cyclic cholecystokinin analogues for their ability to stimulate amylase secretion from isolated pancreatic acini.

Inhibition of CCK-8 induced release of amylase from pancreatic cells was used for measurement of CCK_A antagonism (Yamazaki et al. 1995; Akiyama et al. 1996; Patel et al. 1996; Taniguchi et al. 1996; Ballaz et al. 1997; Martin-Martinez et al. 1997).

Deyer et al. (1993) reported on acetylcholine and cholecystokinin induced acid extrusion in mouse isolated pancreatic acinar cells as measured by the microphysiometer. The microphysiometer continually measures the pH of the medium bathing a cell sample. *EC*₅₀ values for the acidification rate were determined for CCK and CCK analogues. Dunlop et al. (1997) used the Cytosensor microphysiometer to analyze the activity of cholecystokinin antagonists against the CCK-4-mediated response in hCCK-B CHO cells.

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Cholecystokinin Activity (Isolated Rat Pancreatic Acini)

PURPOSE AND RATIONALE

Cholecystokinin (CCK) is one of the first discovered gastrointestinal hormones and one of the most abundant neuropeptides in the brain (Crawley and Corwin (1994). CCK is found in high concentrations in the mammalian brain and may be implicated in the neurobiology of anxiety and panic disorder (Bourin et al. 1996). Two types of CCK receptors have been identified (Wank 1995; Noble et al. 1999; Miyasaka and Funakoshi 2003): CCK-A receptors are mainly located in the periphery, but are also found in some areas of the CNS. CCK-B receptors (Noble and Roques 1999) are widely distributed in the brain. Major biological actions of CCK are the reduction of food intake (Moran and Kinzig 2004) and the induction of anxiety-related behavior. Inhibition of feeding is mainly mediated by the A-type receptors, whereas anxiety-like behavior is induced by stimulating B-type receptors (Fink et al. 1998).

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Receptor Binding of Cholecystokinin

PURPOSE AND RATIONALE

Receptor binding assays for CCK have been described by several authors, such as Innis and Snyder (1980) for rat pancreas; Gaisano et al. (1989), Doi et al. (1990), Blevis et al. (1990), Maletínská et al. (1992) for isolated pancreatic acini; Saito et al. (1981), Chang et al. (1983) for guinea pig cerebral cortex, Praissman et al. (1983) for guinea pig mucosa, Van Dijk et al. (1984) for rat brain and pancreas; Steigerwalt et al. (1984), Chang and Lotti (1986) for gallbladder membranes; Kaufmann et al. (1993) for guinea-pig cerebral cortex and rat pancreas.

Using transfected COS cells, Talkad et al. (1994) identified three different states of the pancreatic CCK receptor, with the very-low-affinity state being the most abundant.

DeTullio et al. (1999) and Herranz (2003) reviewed advances in the chemistry of cholecystokinin receptor agonists and antagonists.

Radioimmunoassays for cholecystokinin have been developed (Harvey 1979) and are available as commercial kits.

PROCEDURE

Membranes of rat (Sprague Dawley) pancreas, guinea pig (Hartley) cerebral cortex, and bovine gallbladder are prepared by homogenization in 50–100 vol of 50 mM Tris-HCl (pH 7.4 at 37°C) using a Polytron (Brinkman, PT 10, setting 4 for 10 s for pancreas or brain and maximal speed for bovine gallbladder). Homogenates are centrifuged at 50,000 g for 10 min, and

the pellets are resuspended in the same buffer and centrifuged as described above. The resulting pellets are resuspended in 4000, 80, and 25 ml of binding assay buffer for each g of original tissue wet weight of pancreas, brain, and gallbladder, respectively. ^{125}I -CCK-8 binding assay buffer contains 5 mM dithiothreitol, 50 mM Tris-HCl (pH 7.4 at 37°C), 5 mM MgCl_2 , 2 mg of bovine serum albumin, and bacitracin at 0.14 mg/ml for pancreas; 10 mM HEPES, 5 mM MgCl_2 , 1 mM EGTA, bacitracin at 0.25 mg/ml, and 130 mM NaCl (pH adjusted to 6.5 with NaOH) for brain; and 10 mM HEPES, 5 mM MgCl_2 , 1 mM EGTA, bacitracin at 0.25 mg/ml, soybean trypsin inhibitors at 0.2 mg/ml, and 130 mM NaCl (pH 6.5) for bovine gallbladder.

^{125}I Bolton-Hunter labeled CCK-8 (New England Nuclear) is used. Free and bound ^{125}I -CCK-8 are separated by filtration using Whatman G/F B glass fiber filters that are presoaked with 50 mM Tris-HCl (pH 7.4) containing bovine serum albumin (1 mg/ml). Immediately after the filtration, the filters are washed rapidly three times with 4 ml of Tris-HCl containing bovine serum albumin (0.1 mg/ml). Radioactivity is counted with a gamma counter, e. g. Beckman Instruments. Specific ^{125}I -CCK-8 binding is defined as the difference between total binding and nonspecific binding in the presence of 1 mM CCK.

EVALUATION

IC_{50} values are determined by regression analysis of displacement curves. Inhibitor constants (K_i) are calculated from the formula

$$K_i = I / (K_d' / K_d - 1)$$

where I is the concentration of the inhibitor, and K_d and K_d' are the dissociation constants of ^{125}I -CCK-8 in the absence and presence of the inhibitor, respectively.

MODIFICATIONS OF THE METHOD

Subtypes of the cholecystokinin receptor, forming a cholecystokinin receptor family, have been described, such as CCK_A from pancreas and other parts of the gastrointestinal tract and in a few discrete brain regions (Kachur et al. 1991; Poirot et al. 1992; Wank et al. 1992a).

According to the IUPHAR Compendium of Receptor Characterization and Classification 1998; CCK_A and CCK_B receptors are designated as CCK_1 and CCK_2 , respectively.

A survey on ligands for cholecystokinin receptors was given by Trivedi (1994).

Van der Bent et al. (1994) described molecular modeling of **CCK-A receptors**.

For CCK_A receptor binding assays (Fossa et al. 1997), the pancreas from a male Hartley guinea pig is dissected and placed in saline. Fatty tissue and blood vessels are dissected away and the tissue placed in 20 vol of buffer (50 mM Tris HCl, pH 7.4, 0.35 mg/ml bacitracin and 0.5 mg/ml soybean trypsin inhibitor) at 4°C and minced using scissors. The tissue is homogenized (Polytron, setting no. 9 for two 15-s bursts), strained through gauze and centrifuged at 100,000 g for 15 min at 4°C. The supernatant is discarded and the pellet resuspended in 20 vol of buffer and recentrifuged. The final pellet is diluted to a concentration of 1.25 mg/ml (original wet weight) in buffer and kept on ice until used. The incubation reaction is initiated by the addition of 100 μl of tissue to 96-well plates containing 150 μl of incubation buffer (50 mM Tris HCl, pH 7.4, and a final concentration of 5 mM MgCl_2 , 5 mM dithiothreitol and 1% DMSO) with 60 pM final concentration of ^{125}I -BH-CCK_{8S}, and drug or vehicle. After a 30-min incubation the reaction is terminated by rapid filtration using a Skatron cell harvester (Skatron Instruments, Inc. VA) onto GF/B filters that were soaked for 2 h in 50 mM Tris HCl, 0.1 mg/ml bovine serum albumin. The filters are dried and counted on a Betaplate counter (Wallac Inc. Gaithersburg, MD) for 45 s per sample.

Povoski et al. (1994) reported cholecystokinin receptor characterization and cholecystokinin A receptor messenger RNA expression in transgenic mouse pancreatic carcinomas and dysplastic pancreas.

Yule et al. (1993), Blevins et al. (1994) recorded intracellular Ca^{2+} concentration signaling stimulated by cholecystokinin or by a partial CCK agonist in Chinese hamster ovary-CCK-A cells or in isolated pancreatic acini.

Ghanekar et al. (1997) established a Chinese hamster ovary cell line bearing the mouse type A cholecystokinin receptor.

CCK_A agonists were evaluated as anorectic agents (Pierson et al. 1997; Simmons et al. 1998).

CCK_B receptors occur predominantly in the central nervous system indicating involvement in behavioral functions (Moran et al. 1986; Wank et al. 1992b; Derrien et al. 1994; Schäfer et al. 1994) but also in pancreatic cancer cells (Smith et al. 1993; Zhou et al. 1992) and small lung cell cancer lines (Sethi et al. 1993).

For CCK_B receptor binding assays (Fossa et al. 1997), guinea pig cortex is homogenized with a Teflon homogenizer in 20 vol of 50 mM Tris HCl (pH 7.4) containing 5 mM MgCl_2 at 4°C and centrifuged at

100,000 *g* for 30 min. The supernatant is discarded and the pellet resuspended and spun again. The pellet is diluted to a concentration of 10 mg/ml (original wet weight) with assay buffer (10 mM HEPES, 5 mM MgCl₂, 1 mM EGTA, 130 mM NaCl, and 0.2 mg/ml bacitracin, pH 6.5) before use. The incubation reaction is initiated by the addition of 50 µl of tissue to 96-well plates containing 150 µl of assay buffer with 1% DMSO final concentration, 50 pM final concentration of ¹²⁵I-BH-CCK_{8S} and the appropriate concentration of drug or vehicle. Non-specific binding is estimated using 1 µM CCK_{8S}. The reaction is terminated by spinning the plates using a H1000B rotor at 3000 rpm for 5 min at 4°C. The pellet is washed with 200 µl of 50 mM Tris HCl and respun. The supernatant is again discarded, the pellet resuspended and the tissues harvested onto Betaplate filters soaked in 0.2% polyethylenimine for 2 h using a Skatron cell harvester (Skatron Instruments, Inc., VA). The filtermats are dried and counted on a Betaplate counter (Wallac Inc. Gaithersburg, MD) for 45 s per sample.

Lee et al. (1993) described cloning and characterization of the human brain cholecystokinin-B/gastrin receptor.

Kaufmann et al. (1993) studied the binding of a series of succinylated cholecystokinin tetrapeptide derivatives to different tissues and their effects on intracellular calcium mobilization ([Ca²⁺]_i) in the human T-cell line Jurkat and rat pituitary (GH3) cells.

Durieux et al. (1989) described [³H]pBC 264 as the first highly potent and very selective radioligand for CCK_B receptors.

Slaninova et al. (1995) recommended the radioiodinated CCK₈ analogue, SNF 8702, as a selective radioligand for CCK_B receptors.

Knapp et al. (1990) found CCK-B receptor heterogeneity in various brain areas of the guinea pig using a highly selective CCK-B receptor radioligand.

Dunlop et al. (1996) described the functional characterization of a Chinese hamster ovary cell line transfected with the human CCK-B receptor gene. Functional coupling in these cells was demonstrated using agonist stimulated mobilization of intracellular Ca²⁺, measured with the FURA-2 technique.

Kaufmann et al. (1995) described the effects of guanyl nucleotides on CCK_B receptor binding in brain tissue and continuous cell lines, such as Jurkat T-cells, rat pituitary GH3 cells, rat glioma C6 cells, and small cell lung cancer NCI-H69 cells.

Cuq et al. (1997) reported that mRNAs encoding CCK_B but not CCK_A receptors are expressed in human T lymphocytes and Jurkat lymphoblastoid cells.

CCK_A receptor antagonists have been described (Chang and Lotti 1986; Makovec et al. 1986; Evans 1993; Gully et al. 1993) as well as **CCK_B receptor antagonists** (Hill and Woodruff 1990; Ohtsuka et al. 1993; Pendley et al. 1993).

Selective non-peptide **CCK_B/gastrin receptor antagonists** have been described by Bertrand et al. (1994, Makovec et al. 1999; Takeuchi et al. 1999). Harper et al. (1999) analyzed some of them in radioligand binding assays in mouse and rat cerebral cortex.

Cholecystokinin dipeptoid antagonists with anxiolytic properties which bind preferably to CCK_B receptors have been reported by Boden et al. (1993).

CCK_A receptor antagonists (Ballaz et al. 1997) and **CCK_B receptor antagonists** (Revel et al. 1998) with anxiolytic-like activity in animal models were described.

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J.7.0.12**Acute Experimental Pancreatitis****PURPOSE AND RATIONALE**

Acute interstitial pancreatitis can be induced in the rat by excessive doses of a pancreatic secretagogue (Lampel and Kern 1987) such as caerulein (Renner et al. 1986; Ito et al. 1991; Yazu et al. 1991).

PROCEDURE**Induction of Pancreatitis**

Male Wistar rats weighing 200 g are used. Pancreatitis is induced by caerulein given as 5 intraperitoneal injections of 40 µg/kg each at hourly intervals on day 1. Controls receive saline injections only. Treatment with potential drugs is started at day 1 and continued for 7 or 14 days. The rats are sacrificed after 1 or 2 weeks following caerulein injection.

Preparation of Pancreatic Acini

The rats are decapitated after overnight fasting and the pancreata are quickly removed. The pancreata are injected with digestion medium containing 170 U/ml collagenase and incubated for 15 min at 37°C. The medium is changed for the second incubation for 60 min with 15 ml digestion medium containing 200 U/ml collagenase. Acini are dissociated by sequential passage through 4 pipettes of different diameters in a standard medium containing 0.1% soybean inhibitor. The suspension is filtered through a single layer of gauze and layered over 15 ml of 4% bovine albumin. After centrifugation for 2 min at 400 rpm, the pellet is washed 3 times with 20 ml of incubation medium containing 0.5 mM CaCl₂. Finally, the acini are resuspended in 10–20 ml of the incubation medium containing 10 mM HEPES, 145 mM NaCl, 4.7 mM KH₂PO₄, 1.0 mM CaCl₂, 1.2 mM MgCl₂, 16.5 mM glucose, 0.1% bovine serum albumin, 0.01% soybean trypsin inhibitor, Eagles minimal essential amino acids and vitamins, adjusted to pH 7.4 and bubbled with 100% oxygen.

Measurement of [³H]-Thymidine Uptake Into Pancreatic Acini

Aliquots (5 ml) of the acini suspension are incubated at 37°C for 30 min, and 6-[³H]-thymidine (5 mCi/mmol) is added as a final concentration of 0.8 mCi/mol. After incubation for 60 min, 200 ml aliquots are filtered by vacuum through Whatman GF/B glass fiber filters. Filters which trapped the acini are washed with 15 ml ice-cold buffer. After adding 5 ml of Aquazol II to

each vial, the radioactivity of the filters is counted with a scintillation spectrometer.

Measurement of Amylase Secretion from Pancreatic Acini

Acini are resuspended in 5 ml of the incubation medium and pre-incubated at 37°C for 30 min. Carbachol at concentrations between 10^{-7} and 10^{-4} M or CCK-8 at concentrations between 10^{-11} and 10^{-8} M is added and the acini suspension is incubated at 37°C for an additional 30 min. Triplicate 300 µl aliquots are centrifuged for 5 s at 3000 rpm. The amylase activity in the supernatant is measured by a commercial amylase test. Triplicate 500 µl aliquots are sampled before adding the secretagogue and the amylase contents (total amylase) are measured after sonification.

EVALUATION

Thymidine uptake is compared between control, caerulein treated animals and animals treated with drugs. Dose-response curves of amylase secretion after carbachol and CCK-8 are established for each group. Moreover, amylase concentrations in the acini are compared.

MODIFICATIONS OF THE METHOD

Griesbacher and Lembeck (1992), Griesbacher et al. (1993) studied the prevention of **caerulein-induced** experimental acute pancreatitis in the rat by the bradykinin antagonist HOE 140.

Several authors (Ito et al. 1994; Ogden et al. 1994; Liu et al. 1995; Sledzinski et al. 1995; Weidenbach et al. 1995; Chen et al. 1996; Lembeck and Griesbacher 1996; Asano et al. 1997; Ito et al. 1997) used different dose regimens of caerulein administration to induce acute pancreatitis in rats.

Niederau et al. (1995) used caerulein to induce acute necrotizing pancreatitis in mice and evaluated the protective effects of secretin and of the cholecystokinin-receptor antagonists proglumide and benztript.

Huch et al. (1995) induced necrotizing pancreatitis by intraductal infusion of low-dose glycodeoxycholic acid (10 mmol/l) followed by intravenous cerulein (6 µg/kg/h) for 6 h.

Several other chemicals and drugs may induced acute pancreatitis (Vogel 1994).

Merkord et al. (1997) studied the pathogenesis and the time course of lesions of acute interstitial pancreatitis in rats induced by intravenous injection of 6 mg/kg **dibutyltin dichloride** (DBTC), an organotin

compound used in chemical industry and in veterinary medicine. First, the cytotoxic effects on the biliopancreatic duct epithelium lead to epithelial necrosis with obstruction of the duct, subsequent cholestasis, and interstitial pancreatitis; and second, the hematogenic effects of DBTC cause direct injury of pancreatic cells followed by interstitial edema and inflammation. A chronic course is found when the obstruction of the duct and cholestasis persist.

Destruction of acinar cells has been found after the administration of **ethionine** in rats (Herman and Fitzgerald 1962).

Niederau et al. (1985), Neuschwander-Tetri et al. (1994), Norman et al. (1995), Van Laethem et al. (1995), Taniguchi et al. (1996) described acute necrotizing pancreatitis induced by caerulein in **mice**.

Pancreatitis can be induced in mice by a **choline-deficient diet** (Lombardi et al. 1975; Niederau et al. 1990). Mice weighing 10–14 g are fed regular laboratory chow ad libitum before the experiment. A choline-deficient diet supplemented with 0.5% ethionine is given for a period of 66 h, after which it is replaced by regular chow.

Lake-Bakaar and Lyubsky (1995), Hirano (1997), Niederau et al. (1995) induced acute pancreatitis in female Swiss-Webster mice by feeding a choline- and methionine-deficient diet supplemented with 1% ethionine.

Emanuelli et al. (1994) demonstrated that a single injection of endotoxin (lipopolysaccharides, *E. coli* 0111-B4) into the superior pancreaticoduodenal artery of **rabbits** induced a dose-dependent acute necrotizing pancreatitis.

Watanabe et al. (1993) induced acute hemorrhagic pancreatitis in rats by surgically **closing a 1 cm length of duodenal loop** at points proximal and distal to the orifice of the pancreatic duct for 6 h with bypassing the bile from the liver hilus distal to the closed loop. The effects of Hoe 140, a bradykinin antagonist, were studied.

Ha et al. (1994, 1996) used the closed duodenal loop technique to study the role of endogenous and exogenous cholecystokinin in experimental pancreatitis and the effect of a cholecystokinin receptor antagonist on the early stage of the healing process in acute pancreatitis.

Kimura et al. (1988) found beneficial effects of a cholecystokinin A receptor antagonist in three methods of acute experimental pancreatitis: pancreatitis in mice induced by 6 intraperitoneal injections of 50 µg/kg caerulein, necrotizing pancreatitis in rats induced by injection of sodium taurocholate into the common

bile duct followed by 4 subcutaneous injections of 50 µg/kg caerulein, and in closed duodenal loop induced pancreatitis in rats.

Obermaier et al. (2001, 2004a, 2004b) described **ischemia/reperfusion-induced pancreatitis** in rats. Male Wistar rats weighing 290–330 g were anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital. They were placed on a heating pad and ventilated after tracheotomy with normal air (TSE, Bad Homburg, Germany). Polyethylene catheters were placed in the right carotid artery and the left jugular vein. The upper abdomen was opened by transverse laparotomy. The intestine was exteriorized to the right. The stomach was turned up cranially, and fixed on the skin by sutures. All exteriorized segments were moistened and covered with transparent foil to prevent drying. The pancreas was separated from the stomach and the short gastric vessels were ligated. The border to the caudal part of the pancreas tail segment was the constant branch of the pancreaticoduodenal artery running to the caudal part of the spleen. Pancreatic and connective tissue in the area of the celiac axis were dissected. Thus the complete vascular isolation of the pancreatic tail pedunculated on the splenic vessels was accomplished.

The common hepatic artery was identified in order to allow PO_2 measurements, the pancreas was fixed on an oval plastic pad with sutures to the connective tissue and splenectomy was performed. A PO_2 -sensitive Clark-type probe was inserted into the pancreatic tissue. The probe was fixed to connective tissue on its distal end with a suture. For local intra-arterial access, a small polyethylene catheter was inserted in the retrograde direction into the left gastric artery and fixed. The pancreatic tail-segment was covered with foil to prevent access of ambient air and drying. After a stabilization period of 10 min, the pancreatic tail segment was flushed with 1 ml of heparinized saline via the catheter in the left gastric artery, after clamping the celiac axis and the common hepatic artery. Paleness of the pancreatic tail segment indicated successful flushing.

For the induction of isolated pancreatic tail ischemia two clamps were put on the splenic vessels and then the clamps of the celiac axis and the common hepatic artery were removed. Continuously decreasing tissue PO_2 indicated successful ischemia. The clamps on the splenic vessels were removed after an ischemic period of 120 min, and disappearing paleness always indicated successful reperfusion. The animals were observed for a further 2 h. At the end of the experiment, the pancreas was excised for histological examination.

Biopsy samples from the non-ischemic head served as control tissue to exclude experimental side-effects on the non-ischemic pancreatic head.

Microcirculatory measurements were done by intravital fluorescence microscopy. Histological examinations of the perfused pancreas tail were performed after staining with hematoxylin and eosin.

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J.7.0.13

Taurocholate-Induced Pancreatitis in the Rat

PURPOSE AND RATIONALE

Retrograde infusion of bile salts into the pancreatic duct induces severe necrotizing pancreatitis indicated by reduced amylase output and histological changes.

PROCEDURE

Male Sprague-Dawley or Wistar rats weighing 200–300 g are anesthetized by i.m injection of a mixture of 87 mg/kg ketamine and 13 mg/kg xylazine. After laparotomy, the pancreaticobiliary duct is cannulated through the duodenal papilla with a polyethylene catheter (P10, Clay Adams) which is introduced by means of a puncture in the duodenum. A precision pump is used to infuse 0.6 ml 5% sodium taurocholate into the pancreaticobiliary duct during a 10-min period at an infusion rate of 6 ml/h. The catheter is then withdrawn and the abdominal cavity surgically closed.

After various time intervals (several hours up to 2 weeks), the animals are anesthetized and the pancreaticobiliary duct is cannulated again. The response to various doses of caerulein is measured.

EVALUATION

The degree of amylase output (mg/h) is taken as parameter. Dose response curves after various doses of caerulein are established.

MODIFICATIONS OF THE METHOD

The effects of various agents on experimental pancreatitis induced by retrograde intraductal injection of **taurocholate** solution or bile acid in rats have been studied by various authors (Bielecki et al. (1994), Hietaranta et al. 1995; Nakae et al. 1995; Niederau et al. 1995; Kimura et al. 1996; Mithofer et al. 1996; Paran et al. 1996; Tachibana et al. 1996; Norman et al. 1997; Plusczyk et al. 1997; Manso et al. 1998).

Tanaka et al. (1995), Sakai (1996) induced necrotizing pancreatitis by retrograde injection of **deoxycholate** solution into the biliopancreatic duct of rats.

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J.7.0.14

Chronic Pancreatitis

PURPOSE AND RATIONALE

Chronic pancreatitis was induced by various means in rats (Zhou 1990, 1994; Goto et al. 1995; Puig-Diví et al. 1996), golden hamsters (Rutishauser et al. 1991, 1995), cats (Reber et al. 1992, 1999; Zhao et al. 1996), dogs (Hayakawa et al. 1993; Tanaka et al. 1994, 1998), and pigs (Vinter-Jensen et al. 1997).

Puig-Diví et al. (1996) induced chronic pancreatitis in rats by trinitrobenzene sulfonic acid infusion into the pancreatic ducts.

PROCEDURE

Male Sprague Dawley rats weighing 300–350 g are anesthetized with ketamine (100 mg/kg i.p.) after an overnight fast. Access to the pancreas is gained through a ventral midline incision. The duodenum is opened and the biliopancreatic duct cannulated through the papilla using polyethylene tubing (PE 10). The biliopancreatic secretion is allowed to drain freely for 15 min. To prevent liver damage, the duct is tied close to the liver. Retrograde infusion is initiated by means of a controlled pressure device that uses the height of a liquid column (trinitrobenzene sulfonic acid or vehicle) in a vertical pipette connected to the infusion cannula to control the intraductal pressure and infusion volume. Ductal pressure is never allowed to exceed 20 cm H₂O. In the treated group 0.4 ml of 2% trinitrobenzene sulfonic acid in phosphate buffered saline + 10% ethanol (pH 8) is infused. Ethanol is employed as an epithelial “barrier breaker” to facilitate trinitrobenzene sulfonic acid penetration into the tissue. Rats in the control group undergo the same procedure, except that trinitrobenzene sulfonic acid is absent from the infusion medium.

The total exposure time of the gland to the instillate is 60 min, followed by a washout period of 30 min. Ligatures are then released, the duodenum and the abdominal wall are sutured, and the animals are kept under observation for 2 h after surgery. Rats are then

transferred to individual cages, where they are fasted for 24 h. Thereafter they receive standard chow and their weight gain is recorded weekly.

Blood is withdrawn after various time intervals by cardiac puncture under light ether anesthesia for determination of glucose and serum α -amylase (EPS test, Boehringer Mannheim GmbH).

Groups of treated rats and controls are sacrificed at 3, 4, and 6 weeks after the surgical procedure. Pancreata are fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Several sections are cut and stained with haematoxylin-eosin for light microscopy evaluation. The degree of periductal and intralobular fibrosis, patchy acute and chronic inflammatory cell infiltrates, common duct stenosis, and segmentary gland atrophy is evaluated.

EVALUATION

Data are expressed as means \pm SEM. A two-tailed Student's *t*-test for unpaired values is used for statistical comparison of mean values of serum amylase, glucose and rat weight.

MODIFICATIONS OF THE METHOD

Chung and Richter (1971), Zhou et al. (1990, 1994) induced chronic pancreatitis in rats by **ligation of the pancreatic duct**.

Injection of oleic acid (Goke et al. 1989; Goldstein et al. 1989; Andersen et al. 1994; Kakugawa 1996; Seymour et al. 1995, 1998) or of a viscous solution of zein – oleic acid – linoleic acid (Kataoka et al. 1998) into the pancreatic duct was used to induce chronic pancreatitis in rats.

Goto et al. (1995) described a chronic pancreatitis model with diabetes induced by intraperitoneal **cerulein** injection plus water immersion stress in rats.

Pancreatic blood flow was measured in **cats** with chronic pancreatitis induced by partial ligation of the pancreatic duct by Austin et al. (1980), Reber et al. (1992, 1999), Widdison et al. (1992).

Zhao et al. (1996, 1998) induced progressing lesions of chronic pancreatitis in cats by intraductal injection of alcohol or by a combination of intraductal and intraparenchymal injection of ethanol together with partial obstruction of the main pancreatic duct to 70% of its original lumen by fixation of a small catheter in the papilla.

Hayakawa et al. (1993) induced pancreatolithiasis in **dogs** by partial obstruction of the major pancreatic duct.

Rats of the diabetic strain WBN/Kob (Tsuchitani et al. 1985; Nakama et al. 1985) develop **sponta-**

neously chronic pancreatitis (Ohashi et al. 1990; Sato et al. 1993; Sugiyama et al. 1996a, b; Arai et al. 1998; Ito et al. 1998) with pancreatic fibrosis and parenchymal destruction and both endocrine and exocrine pancreatic dysfunction (see also K.2.0.2).

Transgenic mice overexpressing TGF β ₁ (Sanvito et al. 1995) develop tissue changes in the pancreas resembling changes found in chronic pancreatitis.

Shetzline et al. (1998) identified target tissues of pancreatic polypeptide using an *in vivo* radioreceptor assay in order to further elucidate the function of this hormone.

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J.8 Liver Function

J.8.1 Hepatocellular Function

J.8.1.1 Hepatitis in Long Evans Cinnamon Rats

PURPOSE AND RATIONALE

The Long Evans Cinnamon strain of rats has been recommended as a useful model to study genetically transmitted fulminant hepatitis and chronic liver disease (Yoshida et al. 1987; Hawkins et al. 1995). The underlying cause is thought to be due to excessive copper accumulation in the liver of Long Evans Cinnamon rats, thus making this animal a model for Wilson's disease in humans (Okayasu et al. 1992). Chelation therapy or feeding a copper-deficient diet can ameliorate the symptoms in Long Evans Cinnamon rats and Wilson's disease (Togashi et al. 1992).

PROCEDURE

Male Long Evans Cinnamon rats obtained from a commercial breeder at an age of 5 weeks are housed in temperature- and humidity-controlled rooms at a 12:12 light/dark cycle. Groups of 6–10 rats are given different diets based on a 15% purified egg protein diet and supplemented with vitamins or drugs. Drugs are applied via minipumps intraperitoneally implanted under ether anesthesia.

The occurrence of jaundice is easily observable as the time when the ears and tail turn yellow and the urine becomes bright orange, staining the fur in the lower abdominal region. Usually, the jaundice progressively worsens, ending in death of the animal within about a week. Incidence of jaundice and mortality vs. time are used as parameters.

EVALUATION

Statistics are performed using StatView II software package for Student's *t*-test, ANOVA, and the Scheffé *F*-test for comparison between means. All data are expressed as means. A *p*-value < 0.05 is used as the threshold of significance.

MODIFICATIONS OF THE METHOD

Several drugs which are known to be effective or which are potentially effective in treatment of Wilson's disease were studied in this animal model, such as D-penicillamine (Togashi et al. 1992; Yokoi et al. 1994; Shimizu et al. 1997), trientine (= triethylenetetramine) (Iseki et al. 1992; Sone et al. 1996; Yamamoto et al. 1997), tetrathiomolybdate (Ogra et al. 1995; Suzuki 1997; Sugawara et al. 1999), or the investigative drug TJN-101 (Yokoi et al. 1995).

The interferon- γ transgenic mouse which carries the mouse INF- γ gene develops chronic hepatitis from the age of 6–10 weeks and was recommended by Okamoto et al. (1999) as a model for **chronic hepatitis**.

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J.8.1.2**Temporary Hepatic Ischemia****PURPOSE AND RATIONALE**

Hepatocellular function is altered by temporary hepatic ischemia as occurring during surgical management of acute hepatic trauma and being essential during hepatic transplantation. To study this, total hepatic ischemia in rats is produced by placing a ligature around the hepatic artery, portal vein, and the common bile duct.

PROCEDURE**Hepatic Ischemia Procedure**

Male albino Holtzman rats, weighing 300–350 g are fasted for 16 h prior to the experiment but are allowed water ad libitum. The rats are anesthetized lightly with ether and the abdominal cavity is opened through a midline incision. Splenectomy is performed following which a temporary extracorporeal splenofemoral shunt is established between the splenic vein and the right femoral vein using a PE-190 tubing. To insure total hepatic ischemia, the portal vein as the hepatic artery and the bile duct are occluded by placing a tourniquet around the vessels. Blood pressure is measured via a catheter inserted into the right femoral artery. After heparinization (10 mg/kg), hepatic ischemia is produced for 60 min. During the ischemic period, 0.7 ml of saline is given i.v. at 20-min intervals for volume replacement. At the end of the 60-min is-

chemic period, the tourniquet around the portal vein, hepatic artery and the bile duct is removed in order to re-establish blood flow to the liver. The abdominal incision is then closed and the animals receive either saline (nontreated) or the drug. Following administration of saline or the drug the catheters are removed and the animals are returned to their home cages.

Sham-operated animals are prepared exactly in the same manner except that the tourniquet around the portal vein, hepatic artery, and the bile duct are not placed.

Measurement of Indocyanine Green Clearance

Three hours following the end of ischemia, the experimental as well as the sham-operated rats are lightly anesthetized with ether and a femoral artery and vein of each animal cannulated. Sodium heparin (400 units) is given i.v. and the animals are allowed to awake. Indocyanine green is given i.v. at 5 mg/kg (low) or 25 mg/kg (high) to the animals via the femoral vein and 0.2 ml arterial blood samples are taken at 5, 6, 8, 10, 12, 15, 18, and 20 min later. The blood samples are diluted with 0.8 ml of 1% bovine serum albumin in normal saline and centrifuged at 6000 rpm for 20 min at 4°C. The spectrophotometric absorbance of the supernatant is read at 800 nm and the indocyanine green concentration determined from a standard curve.

EVALUATION

The $t_{1/2}$ of indocyanine green clearance is computed for each animal using a computer program which calculates the least square line of log indocyanine green vs. time. Mean and standard errors for each group are compared using the Student *t*-test.

MODIFICATIONS OF THE METHOD

Daemen et al. (1989) compared the electromagnetic versus the microsphere and the clearance method for liver blood flow measurement in the rat.

Kawaguchi et al. (2004) described the protective effect of a Rho kinase inhibitor against hepatic ischemia-reperfusion injury in rats.

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J.8.1.3

Model for Direct Transhepatic Studies in Dogs

PURPOSE AND RATIONALE

A chronic conscious dog model for repeated transhepatic studies over a period of 6–8 weeks was developed by O'Brien et al. (1991). This model can be applied to the study of the hepatic effects of pancreatic hormone secretion and glucose metabolism, to studies of the hepatic mechanisms associated with high first-pass metabolism and food interactions of drugs (Semple et al. 1990), and to studies of insulin balance in dogs that have undergone previous pancreatectomy and islet cell auto-transplantation.

PROCEDURE

Four silastic catheters, 0.062 in. ID × 0.125 in. OD (lengths: carotid 70 cm; jugular 70 cm; hepatic 80 cm; and portal 70 cm) and two ultrasonic transit time flow probes suitable for long-term implants (Burton and Gorewit 1984), 4 mm for the hepatic artery and 6 mm for the portal vein, are cleaned with chlorhexidine scrub and rinsed with distilled water. Double velour dacron cuffs are placed 15 cm from the external ends of all the devices.

Male dogs weighing 20–25 kg are sedated and anesthetized with a 2% halothane/L O₂ mixture. Skin interfacial sites and subcutaneous pockets for placement of catheters are prepared. After skin closure the external ends of the catheters are sealed. Then the catheters and flow probes are placed into the abdomen by retrieving them from the subcutaneous pockets. First, the hepatic artery flow probe is placed, then, the portal venous

flow probe inserted. To eliminate extrahepatic blood flow, the gastroduodenal artery is ligated. Then, the portal vein and the hepatic vein catheters are placed. After ensuring catheter patency, the abdomen is closed. Finally, a carotid artery catheter and a jugular venous catheter are placed.

EVALUATION

Blood samples can be withdrawn from the catheters placed into the carotid artery, the right external jugular vein, the portal vein, and the hepatic vein. Blood flow is measured by flow probes in the hepatic artery and the hepatic portal vein.

The following values are measured:

- Plasma flow in the portal vein and plasma flow in the hepatic artery,
- drug concentration in the portal vein, in the hepatic artery, in the hepatic vein, and in the right external jugular vein.

From these data plasma flux in the portal vein, in the hepatic artery, and in the hepatic vein and the interval areas under the curve for these vessels are calculated.

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J.8.2

Liver Cirrhosis and Necrosis

J.8.2.1

General Considerations

Various factors induce liver cirrhosis in man, such as alcoholism, viral hepatitis, intoxications, bile duct disorders, inborn diseases, and others. In the process leading to cirrhosis, accumulation of connective tissue and parenchymal regeneration are competing events. Excessive formation of connective tissue with collagen overproduction in the liver reduces hepatic blood flow, impairs the metabolic functions of the liver, and increases portal vein pressure. These mechanisms result in hepatic failure, esophageal bleeding, portal hypertension and ascites. Therefore, the search for agents to prevent liver cirrhosis is focused on inhibitors of ex-

cessive connective tissue formation in the liver (Kervar et al. 1976; Nolan et al. 1978). The main component of connective tissue formed as a response to chronic injury is collagen. The collagenous fibers consist of triple helical molecules. Their formation depends on the presence of hydrogen bonds involving the post-translationally hydroxylated amino acid hydroxyproline. If the number of hydrogen bonds is reduced due to a decrease of hydroxylated amino acids, the resulting collagen can not form the triple helix and is degraded instead of being deposited in the extracellular matrix.

Insoluble collagen is responsible for most of the mechanical functions of connective tissue, e. g., bone, tendon and skin, being influenced by hormones, desmotropic drugs, such as D-penicillamine, and by maturation and age (Vogel 1969, 1972, 1974a, b, 1976, 1978, 1980, 1989; Bickel et al. 1990, 1991). The aim of fibrosuppressive compounds is to reduce only the excessive formation of insoluble collagen in the liver leaving collagen synthesis and turnover in other tissues intact. Fibrosuppressive effects by inhibition of proline hydroxylation can be screened with *in vitro* methods, however, the desired organ specificity has to be tested in models of liver cirrhosis and fibrosis *in vivo* together with functional studies of the connective and supporting tissue. Detailed description of methods for studying collagen metabolism and mechanical function of connective tissue is given in the Sect. M.2.1.3 (hormones).

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J.8.2.2

Inhibition of Proline Hydroxylation

PURPOSE AND RATIONALE

The thermal stability of the triple helix of collagenous proteins is crucially dependent upon the intramolecular hydrogen bonds involving the 4-hydroxyproline residues synthesized by the enzyme prolyl 4-hydroxylase. This makes the enzyme a possible target for therapeutic antifibrotic agents.

PROCEDURE

Enzyme activity is assayed in sealed test tubes. The reaction volume of 1 ml contains 50 mM Tris buffer, pH 7.5, 10–100 mM (60000 d.p.m.) 2-oxo[1-¹⁴C]glutarate, 1–50 mM FeSO₄, 0.1–1 mM ascorbate, 10–100 mg (Pro-Pro-Gly)₁₀, 0.1 mg catalase, 2 mg bovine serum albumin, 100 mM dithiothreitol, 0.05–0.2 mg enzyme, and inhibitors in various concentrations. After incubation at 37°C for 30 min, the generated ¹⁴CO₂ is trapped and determined.

EVALUATION

Inhibition modes are determined by plotting 1/v versus 1/concentration of the variable substrate (Lineweaver-Burk plot). The K_i values are derived from a secondary transformation (slopes or intercepts vs. inhibitor concentrations). The lines of best fit for primary plots and secondary transformations are calculated by using the method of least squares. The mean K_i value of 4–6 experiments is calculated.

MODIFICATIONS OF THE METHOD

The collagen hydroxylases lysyl hydroxylase and prolyl 3-hydroxylase have similar reaction mechanisms as prolyl 4-hydroxylase, differing only in the specificity for the amino acid sequence of the substrate (Kivirikko and Myllylä 1982). Instead of (Pro-Pro-Gly)₁₀, 50–500 mg Arg-Ala-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly are used.

The activity of prolyl 3-hydroxylase is assayed in a reaction volume of 1.5 ml containing 50 mM Tris buffer, pH 7.5, 1,000,000 d.p.m. of biologically obtained [2,3-³H]proline-labeled protocollagen substrate in which all proline residues recognized by proline 4-hydroxylase are converted to hydroxyproline, 2 mM ascorbate, 0.2 mg/ml catalase, 2 mg/ml bovine serum albumin, 15 mM 2-oxoglutarate, 50 mM Fe²⁺, 100 mM dithiothreitol, 0.2–2 mg enzyme, and inhibitors at various concentrations. After incubation at 37°C for 30 min, the reaction is stopped by addition of 0.5 ml 10% trichloroacetic acid (w/v). The reaction mixture is then distilled, and 1.6 ml of ³H₂O is counted for radioactivity.

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J.8.2.3

Influence on Collagen Synthesis in Human Skin Fibroblasts

PURPOSE AND RATIONALE

Secretion of collagen by fibroblasts and other cells capable of synthesizing extracellular matrix is dependent on the hydroxylation of proline residues by prolyl 4-

hydroxylase. This enzyme is located in the cisternae of the endoplasmic reticulum. An agent aimed at inhibition of this enzyme must therefore pass both the external cell membrane and the endoplasmic reticular membrane. Organ specificity of a prolyl 4-hydroxylase inhibitor can be achieved by applying a prodrug which can be converted to the active agent only in cells of specialized tissues, e. g., in the liver, but not generally in fibroblasts.

PROCEDURE

Confluent cultures of human skin fibroblasts are preincubated for 24 h at 37°C without serum in glutamine-free Dulbecco's minimal essential medium supplemented with 50 mg/ml sodium ascorbate, 60 mg/ml 3-aminopropionitrile, and 100 U/ml penicillin G. The cells are then exposed to the potential inhibitor at various concentrations for 20 min, followed by the addition of 2 mCi [U-¹⁴C]proline/ml. The incubation is continued for 5 h at 37°C. Then, the cells are separated from the medium. After removal of non-incorporated [¹⁴C]proline, the proteins from medium and cells are hydrolyzed, and the hydroxyproline content is determined by amino acid analysis. The total incorporation of radioactivity serves as marker for protein synthesis.

EVALUATION

Two individual samples are taken for each concentration of the inhibitor and 6 samples for the controls. Proline incorporation is expressed as % of control radioactivity. Hydroxyproline synthesis is expressed as relative Hyp/Pro ratio according to the formula:

$$\left(\frac{\text{Hyp}/\text{Pro}_{\text{sample}}}{\text{Hyp}/\text{Pro}_{\text{control}}}\right) \times 100.$$

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branes readily and are converted intracellularly to the active agent. Proinhibitors which are cleaved hydrolytically can suppress collagen synthesis in chicken calvaria.

PROCEDURE

Calvaria are removed from chicken embryos, age 15 days, and washed for 3 min with DMEM at 37°C. They are then transferred into Pyrex tubes (8–10 calvaria/tube) containing 3 ml medium supplemented with 2 mM glutamine, 6 mCi [U-¹⁴C]-proline, and various concentrations of the inhibitor. The samples are incubated for 1.5 to 6 h at 37°C. The experiment is terminated by placing the tubes in ice and separation of calvaria from the culture medium. The calvaria are washed once with 3 ml of fresh medium, which is then pooled with the incubation medium. Bovine serum albumin and phenyl methyl sulfonyl fluoride are added (final concentration 1 mg/ml and 6 mg/ml, respectively). The calvaria are extracted for 16 h with 25 ml of 0.5 M acetic acid.

The following procedure is identical for the medium and the calvaria extracts. The samples are extensively dialyzed against 0.5 M acetic acid at 4°C. Aliquots are withdrawn for SDS-PAGE1 and the triple-helix stability assay is performed. The remaining material is lyophilized, resuspended in 2 ml 6 N HCl and hydrolyzed at 105°C for 24 h. After evaporation of the acid, the samples are dissolved in 2 ml H₂O, and the hydroxyproline content is determined according to Juva and Prockop (1967).

In order to study the degree of collagen hydroxylation and proportion of collagen biosynthesis, calvaria are incubated in the presence of 10 mCi [3,4-³H]-proline/ml and 2 mCi [U-¹⁴C]-proline/ml for 3 h under the conditions described above. After lyophilization, aliquots of media and calvaria samples are digested with collagenase according to Peterkofsky et al. (1982). The degree of hydroxylation is calculated from the ³H/¹⁴C ratio in the digested material; the amount of collagen as a proportion of total protein synthesis is determined by the relation of collagenase degradable vs. collagenase resistant radioactivity. The stability of the extracted collagenous material against digestion by a trypsin/chymotrypsin mixture is tested according to the procedure proposed by Bruckner and Prockop (1981): An aliquot of either culture medium or calvaria extract is incubated for 15 min at a temperature between 25°C and 45°C in a total volume of 800 μl of 0.04 M NaCl/0.1 Tris, pH 7.4. After quenching to 0°C the sample is digested with 200 μl of a mixture of each 1 mg trypsin and chymotrypsin/ml buffer

J.8.2.4

Influence on Collagen Synthesis in Chicken Calvaria

PURPOSE AND RATIONALE

To find fibrosuppressive agents for therapeutic use, it is necessary to have prodrugs which cross cell mem-

for 15 min at room temperature. One hundred μl of 1 mg BSA/ml buffer is added, and the protease-resistant radioactivity, consisting of triple-helical collagen, is precipitated with 100 μl 100% trichloroacetic acid (w/v). The sample is transferred in total to a Schleicher and Schüll OE 67 filter paper of 2.5 cm diameter. The digested material is removed by repeated washing with cold 5% trichloroacetic acid and methanol. The filters are then dried and the radioactivity is determined. Unhydrolyzed samples are studied by SDS-polyacrylamide gel electrophoresis, and autofluorography. The morphologic appearance of the control and treated cultures is studied by electron microscopy.

EVALUATION

IC_{50} -values of hydroxyproline synthesis are read graphically from concentration response curves. Total protein synthesis is estimated as the incorporation of proline; the mean \pm standard deviation is calculated from four samples.

MODIFICATIONS OF THE METHOD

Canalis et al. (1977) used cultured calvaria from 21 day fetal rats to study the effects of insulin and glucagon on bone collagen synthesis.

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J.8.2.5

Allyl Alcohol Induced Liver Necrosis in Rats

PURPOSE AND RATIONALE

Administration of allyl alcohol induces focal liver necrosis in rats which can be partially prevented by treatment with several drugs such as antibiotics.

PROCEDURE

Female Wistar rats weighing 120–150 g are used. At 8:00 A.M. of the first day food but not water is withdrawn. At 3:00 P.M. the compounds to be tested for

protective activity are administered i.p. or orally. One hour later, the animals are dosed orally with 0.4 ml/kg of a 1.25% solution of allyl alcohol in water. At 8:00 A.M. of the second day the treatment with the potentially protective drugs is repeated. Food but not water is withheld until the third day. At 8:00 A.M. of the third day the animals are sacrificed and the liver removed. The parietal sides of the liver (left, medium and right lobe and lobus caudatus) are checked using a stereomicroscope with 25 times magnification. Focal necrosis is observed as white-green or yellowish hemorrhagic areas clearly separated from unaffected tissue. The diameter of the necrotic areas is determined using a ocular-micrometer. These values are added for each animal to obtain an index for necrosis.

EVALUATION

Using 10 animals for controls and for each treatment group, the mean of necrosis index is calculated and compared with Student's *t*-test. The protective effect is expressed as percentage decrease of the necrosis index versus controls.

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J.8.2.6

Carbontetrachloride Induced Liver Fibrosis in Rats

PURPOSE AND RATIONALE

Chronic administration of tetrachloride to rats induces severe disturbances of hepatic function together with histologically observable liver fibrosis.

PROCEDURE

Groups of 20 female Wistar rats with a starting body weight of 100–150 g are used. The animals are treated orally twice a week with 1 mg/kg carbontetrachloride, dissolved in olive oil 1:1, over a period of 8 weeks. The animals are kept under standard conditions (day/night rhythm 8:00 A.M. to 8:00 P.M., 22°C room temperature, standard diet, e. g. Altromin 1321 pellets, and water ad libitum). Twenty animals serve as controls receiving olive oil only, 40–60 animals receive the carbontetrachloride only, and groups of 20 rats receive in addition the compound under investigation in various doses by gavage twice daily (with the exception of the weekends, when only one dose is given) on the basis

of the actual body weight. The animals are weighed weekly.

At the end of the experiment (8 weeks), the animals are anesthetized and exsanguinated through the caval vein.

In the *serum*, the following parameters are determined:

- Total bilirubin,
- total bile acids,
- 7S fragment of type IV collagen,
- procollagen III N-peptide.

The following organs are prepared for determination of hydroxyproline:

- Liver,
- kidney,
- aortic wall, and
- tail tendons.

The specimens of the organs are weighed and completely hydrolyzed in 6 N HCl. Hydroxyproline is measured by HPLC and expressed as mg/mg wet weight of the organs.

To measure **mechanical properties of connective tissue**, the following organs are prepared: Femur and tibia of both sides, tail tendons, and strips from dorsal skin (Bickel et al. 1990, 1991; detailed description of the methods see N.2.1.3). Furthermore, the influence on the healing process of skin wounds is studied (Method see N.2.1.3.5).

For **histological analysis**, 3–5 pieces of the liver weighing about 1 g are fixed in formalin and Carnoy solution. Three – 5 sections of each liver are embedded, cut and stained with azocarmine aniline blue (AZAN) and evaluated for the development of fibrosis using a score of 0–IV.

| | |
|------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Grade 0: | Normal liver histology. |
| Grade I: | Tiny and short septa of connective tissue without influence on the structure of the hepatic lobules. |
| Grade II: | Large septa of connective tissue, flowing together and penetrating into the parenchyma. Tendency to develop nodules. |
| Grade III: | Nodular transformation of the liver architecture with loss of the structure of the hepatic lobules. |
| Grade IV: | Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and with development of scars. |

EVALUATION

For detection of significant differences ($p < 0.05$), the unpaired t -test is used. For comparison of the scores in the histological evaluation, the χ^2 -test is used.

MODIFICATIONS OF THE TEST

Instead of chronic intoxication with carbontetrachloride resulting in liver fibrosis, acute hepatocellular damage can be achieved by short term application of carbontetrachloride.

Wistar rats with a starting body weight of 120–150 g are treated daily for 5 days with various oral doses of the compound under investigation. From day 2 to day 5 (4 applications) the rats receive by gavage a dose of 1 mg/kg carbontetrachloride dissolved in olive oil (1:1). Blood is withdrawn every day and the aminotransferases ALAT and ASAT, as well as total bilirubin are determined in the serum.

Niederberger et al. (1995, 1998) induced liver cirrhosis and ascites in rats by treatment with carbon tetrachloride.

Sakamoto et al. (2005) induced liver cirrhosis in rats by intraperitoneal injection of 10 mg/kg dimethylnitrosamine three times a week for 3 weeks.

Kawaura et al. (1993) produced liver cirrhosis with ascites in **dogs** by administration of 2 ml carbontetrachloride per kg body weight once a week for 4 weeks. Eight weeks afterwards, the supradiaphragmatic inferior vena cava was constricted to 50% resulting in ascites formation of 500 to 1000 ml. The dogs could be treated by ligation of the common hepatic artery and hepatocyte inoculation into the spleen.

Wirth et al. (1997) studied the effects of a bradykinin B₁ receptor antagonist in rats with CCl₄-induced liver cirrhosis.

In the model of CCl₄-induced liver fibrosis, the antifibrotic agents HOE077 and safronil decreased collagen accumulation in the liver by inhibition of stellate cell activation, which was more pronounced in female than it was in male rats (Wang et al. 1998). In the same model (Bickel et al. 1998) demonstrated powerful antifibrotic effects of a new prolyl 4-hydroxylase (P4-H) inhibitor, S4682, having a K_i of 155 nM at the purified enzyme P4-H. Inhibition of prolyl hydroxylation in stellate cells was inhibited with an IC₅₀ of 39 μ M by S4682. In drug-treated rats, ascites was significantly lower compared with controls. Histological examination proved a significantly lesser score of liver fibrosis after drug treatment.

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J.8.2.7

Bile Duct Ligation Induced Liver Fibrosis in Rats

PURPOSE AND RATIONALE

Bile duct ligation in rats induces liver fibrosis which can be evaluated by histological means and by determination of serum collagen parameters.

PROCEDURE

Male Sprague Dawley rats weighing approximately 250 g are anesthetized with ketanest (Rompun). La-

parotomy is performed under antiseptic conditions. A mid-line incision in the abdomen is made from the xiphosternum to the pubis, exposing the muscle layers and the linea alba, which is then incised over a length corresponding to the skin incision. The edge of the liver is then raised and the duodenum pulled down to expose the common bile duct, which pursues an almost straight course of about 3 cm from the hilum of the liver to its opening into the duodenum. There is no gallbladder, and the duct is embedded for the greater part of its length in the pancreas, which opens into it by numerous small ducts. A blunt aneurysm needle is passed under the part of the duct selected, stripping the pancreas away with care, and the duct is divided between double ligatures of cotton thread. The peritoneum and the muscle layers as well as the skin wound are closed with cotton stitches.

The animals receive normal diet and water ad libitum throughout the experiment. Groups of 5–10 animals receive the test compound in various doses or the vehicle twice daily for 6 weeks. Then, they are sacrificed and blood is harvested for determination of bile acids, 7S fragment of type IV collagen, and procollagen III N-peptide. The liver is used for histological studies and for hydroxyproline determinations. Control animals show excessive bile duct proliferation as well as formation of fibrous septa. The picture is consistent with complete biliary cirrhosis.

EVALUATION

For detection of significant differences ($p < 0.05$), the unpaired *t*-test is used.

MODIFICATIONS OF THE METHOD

Alpini et al. (1994) found an upregulation of secretin receptor gene expression in rat cholangiocytes after bile duct ligation.

Fiorucci et al. (2003) used 4-week bile-duct-ligated cirrhotic rats to study the effects of a nitric oxide-releasing derivative of ursodeoxycholic acid.

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J.8.2.8

Galactosamine Induced Liver Necrosis

PURPOSE AND RATIONALE

Single dose or a few repeated doses of D-galactosamine cause acute hepatic necrosis in rats (Decker and Keppler 1972). Prolonged administration leads to cirrhosis (Lesch et al. 1970).

PROCEDURE

For induction of acute experimental hepatotoxicity, divided doses of 100 to 400 mg/kg D-galactosamine are injected to rats i.p. or i.v. during one day.

For induction of liver cirrhosis, male Wistar rats weighing 110–180 g are injected intraperitoneally three times weekly with 500 mg/kg D-galactosamine over a period of one to 3 months. Potential protective substances are administered orally with the food or by gavage every day. The rats are sacrificed at various time intervals and the livers obtained by autopsy.

EVALUATION

The livers are evaluated by light microscopy and immunohistology using antibodies against macrophages, lymphocytes and the extracellular matrix components, e. g., laminin, fibronectin, desmin, collagen type I, III, and IV. The extent of liver cell necrosis and immunoreactivity for macrophages, lymphocytes and the extracellular matrix components is graded semiquantitatively on a 0 to 4+ scale (0 = absent, 1+ = trace, 2+ = weak, 3+ = moderate, and 4+ = strong). Furthermore, serum enzyme activities, such as GOT and GPT, are determined.

MODIFICATIONS OF THE METHOD

Other agents used to induce experimental cirrhosis are ethionine, thioacetamide (Dashti et al. 1996), dialkyl-nitrosamines, tannic acid, aflatoxins, pyrrolidizine alkaloids, and hepatotoxic components from mushrooms, such as amatoxins and phallotoxins (Zimmerman 1976).

Bruck et al. (1996) found an inhibition of thioacetamide-induced liver cirrhosis in rats by a nonpeptidic mimetic of the extracellular matrix-associated Arg-Gly-Asp epitope.

Intrahepatic cholestasis can be induced by alpha-naphthylisothiocyanate in rats (Krell et al. 1982).

Fulminant liver destruction can be induced with anti-Fas antibody.

Rodriguez et al. (1996) injected inbred female C57BL mice with anti-Fas antibody. The animals died within a few hours due to massive apoptosis of hepatocytes. This was accompanied by the sequential activation of cysteine proteases of the interleukin₁ β -converting enzyme (ICE) and CPP32 (caspase-3) types in the cytosol of the hepatocytes. Systemic injection of the tripeptide *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone inhibited the intracellular activation of CPP32-like proteases *in vivo* and fully protected mice against Fas-mediated fulminant liver destruction and death.

Suzuki (1998) showed that the CPP32 subfamily, rather than the ICE subfamily, plays the dominant role in Fas antibody-induced hepatitis.

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J.8.2.9

Liver Fibrosis Induced by Schistosome Cercariae

CCl₄-induced liver fibrosis is the most commonly used model in liver fibrosis. The major shortcoming of this method is the high relative variability of the amount of collagen produced in the liver by treatment with the toxin. Determination of hydroxyproline, as a parameter of collagen deposition in the liver, showed a large relative variability of 75% after 8 weeks of treatment with twice-weekly administration of CCl₄. To ensure adequate statistical interpretation, 50 rats/group are needed (Bickel et al. 1996).

Exposure of mice tails to *Schistosoma cercariae* causes infiltration of the cercariae into the circulation and subsequent deposition of the schistosome eggs in the liver. Thereafter liver fibrosis is produced in the liver in a time-dependent fashion. Hydroxyproline of the liver increases by threefold and sevenfold, 10 and 18 weeks after infection. The relative variabilities of the hydroxyproline content of the liver were 10% to 20%, 10 and 18 weeks after infection, respectively (Phillips et al. 1977, 1987). The advantages of this model are the simplicity of fibrosis induction, the uniform generation of liver fibrosis with a very low variability with regard to the collagen produced in the liver, and, most of all, that it represents a disease model which accurately corresponds to the human disease. One should not forget that at least 200 million people suffer from schistosomiasis, most of them living in developing countries.

In this model the antifibrotic active compound Saffronil caused a significant reduction of collagen type I and fibronectin in the liver. The beneficial effects of Saffronil were at least in part due to a decrease in stellate cell activation by a mechanism sensitive to tumour growth factor (TGF)_{β1} (Phillips et al. 1997).

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J.9

Eviscerated Animals

J.9.1

Evisceration in Rats

PURPOSE AND RATIONALE

Eviscerated animals have been used to study the influence of hormones and other drugs on carbohydrate disposal (Russell 1942; Creutzfeldt et al. 1961; Wick and Drury 1963; Willms et al. 1969; Tanira and Furman 1999).

“Functional” evisceration has been performed by most authors (Russell 1942; Creutzfeldt et al. 1961; Creutzfeldt and Deuticke 1962; Willms et al. 1969; Penhos et al. 1970; Smith 1986; Smith et al. 1990; Tanira and Furman 1999). The abdominal viscera, with the exception of the liver, are removed, and the blood vessels supplying the liver – the coeliac axis and the portal vein – are tied and cut.

PROCEDURE

Male Wistar rats weighing 180 to 280 g are allowed free access to food and water until the experiment. They are anesthetized with 55 mg/kg pentobarbitone sodium i.p. Cannulae are placed in the left femoral vein and in the abdominal aorta via the left femoral artery.

The abdominal cavity is opened and the anterior mesenteric artery, posterior mesenteric artery and the coeliac axis are cut between double ligatures close to the abdominal aorta. The hepatic portal vein is ligated and cut in the same manner. After ligating and cutting the esophagus and the rectum, the abdominal portion of the gastrointestinal tract is removed, together with the pancreas and the spleen. The muscle and the skin incisions are sutured. After completion of the operation the rats are left for 15 min to equilibrate.

Drugs are injected intravenously through the venous cannula. Blood samples are withdrawn from the aortic cannula before and at various intervals after drug ad-

ministration to be assayed for insulin, blood glucose and non-esterified fatty acids.

EVALUATION

All results are expressed as mean \pm SEM. Statistical analysis is performed using ANOVA.

CRITICAL ASSESSMENT OF THE METHOD

The described "functional" evisceration can be performed within 2–3 min without any hemorrhage or any signs of shock, even in hypophysectomized or adrenalectomized animals. That the liver remaining *in situ* is effectively cut off from the blood stream is proven by the fact that rats eviscerated in this way will die within 1–2 h without infusion of glucose.

MODIFICATIONS OF THE METHOD

Some authors removed also the kidneys and the adrenals in rats (Creutzfeldt and Deuticke 1962).

Dogs were eviscerated and nephrectomized under sodium pentobarbital anesthesia by Levine et al. (1950).

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J.9.2

Evisceration in Rabbits

PURPOSE AND RATIONALE

Evisceration in rabbits was performed by Drury (1935), Wick and Drury (1963), Lippmann and Hommel (1968, 1969), Menzel and Haupt (1972). A detailed description of the technique was given by Lippmann and Hommel (1968).

PROCEDURE

Rabbits of both sexes weighing 2.6–3.2 kg kept on standard diet and tap water ad libitum are used. Food is withdrawn 12 h before surgery and the abdominal skin is shaved. The animals are anesthetized with 50 mg/kg hexobarbital sodium intravenously. After fixation of the animal, the abdominal cavity is opened by electrocautery from the xyphoid process until 3 cm cranial of the symphysis. The rectum is cut between double ligatures as far caudally as possible. Bleeding from the arteria rectalis has to be avoided. The arteria mesenterica inferior, which is a tiny vessel in the rabbit, is ligated close to the aorta. The root of the mesentery is cut up to the superior mesenteric artery which is tightly ligated. Then the arteria coeliaca is ligated in the same manner allowing complete removal of the root of the mesentery. The esophagus is ligated close to the cardia. After double ligature of the portal vein the whole gastrointestinal tract including the pancreas and the spleen is isolated and the liver is functionally cut off. To eliminate the function of the kidneys, the renal arteries are ligated close to the aorta and the ureters close to the hilus. Cannulae are introduced into the caval vein via the renal veins. The right renal vein is ligated close to the hilus and the tip of a polyethylene catheter placed 4–6 cm caudal from the inferior caval vein for blood withdrawal; the left renal vein is also ligated close to the hilus and the tip of a polyethylene catheter placed 6 cm cranial from the inferior caval vein for infusion.

The animal receives via the catheter 750 IU heparin, 0.3 mg/kg lobeline hydrochloride, 3 mg/kg pentylenetetrazole, 0.01 mg/kg ouabain, and 1.3 mg/kg synephrine to stabilize the cardiovascular function.

Drugs are injected intravenously through the infusion cannula. Blood samples are withdrawn from the other cannula before and at various intervals after drug administration to be assayed for insulin and blood glucose.

EVALUATION

All results are expressed as mean \pm SEM. Statistical analysis is performed using ANOVA.

MODIFICATIONS OF THE METHOD

Schäfer (1990) measured glucose disappearance rates into peripheral tissues *in vivo* in adult eviscerated, nephrectomized and adrenalectomized rabbits, Wistar rats and non-diabetic sand rats with physiological insulin concentrations in the blood.

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J.10**Safety Pharmacology of Gastrointestinal Drugs**

See Hering (2006).

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Chapter K

Antidiabetic Activity¹

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K.1 Methods to Induce Experimental Diabetes Mellitus

K.1.1 Pancreatectomy in Dogs

GENERAL CONSIDERATIONS

Dysfunction of the visceral tract has been considered for a long time to be the cause of diabetes mellitus. Bomskov in 1910 reported severe diabetic symptoms in dogs after cannulation of the ductus lymphaticus. This observation, however, could not be confirmed in later experiments (Vogel 1963). The technique was similar to that described by Gryaznova (1962, 1963) for ligation of the thoracic duct in dogs.

Von Mehring and Minkowski in 1890 noted polyuria, polydipsia, polyphagia, and severe glucosuria following removal of the pancreas in dogs. The final proof for the existence of a hormone in the pancreas was furnished by Banting and Best (1922) who could reduce the elevated blood sugar levels in pancreatectomized dogs by injection of extracts of the pancreatic glands. The role of the pituitary gland in development of diabetes has first been elucidated by Housay (1930, 1931) in pancreatectomized dogs (Survey by Beyer and Schöffing 1986).

PURPOSE AND RATIONALE

The technique of complete pancreatectomy in the dog as described in detail by Foà (1971) and by Sirek (1986) has been used by many scientists as a relevant animal model for insulin-deficient diabetes mellitus in man. Some remarks of our own experiences are added to an abbreviated version of Sirek's description.

PROCEDURE

Male Beagle dogs weighing 12–16 kg are used. After appropriate premedication the animal is placed on its back intubated with a tracheal tube and anaesthesia is achieved and maintained by an inhalation anaesthetics (e.g. fluroane). After removal of the fur and disinfection of the skin a midline incision is made from the xyphoid process reaching well below the umbilicus. Bleeding vessels are ligated and the abdomen is entered through the linea alba. The falciform ligament is carefully removed and the vessels ligated. A self-retaining retractor is applied. By passing the right hand along the stomach to the pylorus, the duodenum with the head of the pancreas is brought into the operating field. First, the mesentery at the unicate process is cut and the process itself is dissected free. The glandu-

lar tissue is peeled off from the inferior pancreaticoduodenal artery and vein. The vessels themselves are carefully preserved. Along a line of cleavage which exists between the pancreas, the pancreaticoduodenal vessels and the duodenal wall, the pancreas is separated from the duodenum and from the carefully preserved pancreaticoduodenal vessels. The small vessels to the pancreas are ligated. The dissection is carried out from both sides of the duodenum. In the area of the accessory pancreatic duct the glandular tissue being attached very firmly has to be carefully removed in order to leave no residual pancreatic tissue behind.

The pancreatic duct is cleaned, doubly ligated and cut between the ligatures. The dissection proceeds until one encounters a small lobe containing the main pancreatic duct. The glandular tissue adheres here firmly to the duodenum. Blunt dissection and ligation of the vessels is followed by ligation of the pancreatic duct. By pulling on the pylorus and the stomach, the pyloric and the splenic parts of the pancreas are delivered into the wound. The duodenal part is placed back into the abdominal cavity. The mesentery of the body and tail of the pancreas is cut with scissors. The small vessels are doubly ligated and cut. The pancreatic tissue is bluntly dissected from the splenic vessels. The pancreatic branches of the splenic vessels are doubly ligated and cut. Working in direction from the spleen to the pylorus, the pyloric part of the pancreas is the last one to be dissected. Finally, all pancreatic tissue is removed.

The surgical field is checked once more for pancreatic remnants. The concavity of the duodenum and its mesentery is approximated by a few silk stitches and the omentum is wrapped around the duodenum. Retroperitoneal injection of 5 ml 1% procaine solution is given to prevent intussusception of the gut. 250,000 IU penicillin G in saline solution are instilled into the peritoneal cavity. The abdominal wall and the subcutaneous layer are closed by sutures and finally the skin is sutured with continuous everting mattress stitches.

After the operation, the animal receives via a jugular vein catheter for 3–4 days the following treatment: 1000 ml 10% glucose solution with 10 IU human insulin Regular, 3 ml 24% Borgal (sulfadioxin/trimethoprim) solution, 2 ml 50% metamizol and 400 IU secretin. On the third day, the animal is offered milk. After the animal has passed the first milk feces, commercially available dry food together with a preparation of pancreatic enzymes for substitution. Insulin is substituted with a single daily subcutaneous dose of a long-acting insulin (e.g. Lantus) and the

insulin dose is individually adjusted according to the blood glucose levels achieved. Vitamin D₃ is given every three months as a intramuscular injection of 1 ml Vigantol forte.

MODIFICATIONS OF THE METHOD

Experiments performed by Houssay (1930, 1931) performing hypophysectomy in pancreatectomized dogs revealed amelioration of the diabetic state. These experiments contributed to the understanding of hormonal control in diabetes mellitus.

Rappaport and coworkers (1966) and Lau and coworkers (1976) used a pedunculated subcutaneous autotransplant of an isolated pancreas remnant for the temporary deprivation of internal secretion in the dog.

Subtotal pancreatectomy in **rats** was described by Scow (1957), Scow and coworkers (1957), Wagner and Cardeza (1957), Bonner-Weir and coworkers (1983), Noguchi and coworkers (1994), Tanigawa and coworkers (1997). The pancreatic tissue between the common bile duct, duodenal loop, and portal vein in the duodenal segment, along the greater curvature in the gastric segment, and along the splenic vein in the splenic segment, was surgically excised in 3- to 4-weeks old rats weighing 80–100 g.

Greeley (1937) proposed a 3-stage procedure with 3–4 weeks intervening between operations for pancreatectomy in **rabbits**.

Itoh and Maki (1996) reported surgical removal of 90% of pancreatic tissue in 7 or 13 weeks old **mice**. Under sodium pentobarbital anesthesia (65 mg/kg i.p.), the pancreas and the spleen were surgically removed with careful conservation of the common bile duct and major vessels surrounding the duodenum. Approximately 10% (by weight and by insulin content) of the pancreas tissue was left intact adjacent to the lower duodenal loop.

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K.1.2

Alloxan-Induced Diabetes

PURPOSE AND RATIONALE

Surveys on chemically induced diabetes in animals were given by Frerichs and Creutzfeldt (1968, 1971). This kind of diabetes predominantly based on insulin-deficiency due to chemically (beta cell toxicity) destroyed pancreatic beta-cells.

Hyperglycemia and glucosuria after administration of alloxan has been described in several species, such as in dogs (Brunschwig et al. 1943, Tasaka et al. 1988), in rabbits (Baily and Baily 1943), in rats (Dunn and McLetchie 1943, Goldner and Gomori 1944) and in other species (Frerichs and Creutzfeldt 1968, 1971).

Guinea pigs have been found to be resistant (Maske and Weinges 1957). Dosage and treatment regimen have been elaborated for the most frequently used species. In most species a triphasic time course is observed: an initial rise of glucose is followed by a decrease, probably due to depletion of islets from insulin, again followed by a sustained increase of blood glucose.

PROCEDURE

Rabbits weighing 2.0 to 3.5 kg are infused via the ear vein with 150 mg/kg alloxan monohydrate (5.0 g/100 ml, pH 4.5) for 10 min resulting in 70% of the animals to become hyperglycemic and uricosuric. The rest of the animals either die or are only temporarily hyperglycemic (Baily and Baily 1943, Pincus et al. 1954, Bänder et al. 1969).

Rats of Wistar or Sprague-Dawley strain weighing 150–200 g are injected subcutaneously with 100–175 mg/kg alloxan (Blum and Schmid 1954, Katsumata and Katsumata 1990, Katsumata et al. 1993).

Male Beagle dogs weighing 15–20 kg are injected intravenously with 60 mg/kg alloxan. Subsequently, the animals receive daily 1000 ml 5% glucose solution with 10 IU Regular insulin for one week and canned food ad libitum. Thereafter, a single daily dose of a long-acting insulin (e. g. Lantus) is administered subcutaneously and the insulin dose is individually adjusted according to the blood glucose levels achieved. (Brunschwig at al 1943, Geisen 1988).

MODIFICATIONS OF THE METHOD

Kodoma and coworkers (1993) described a new diabetes model induced by neonatal alloxan treatment in rats. Male Sprague Dawley rats 2, 4, or 6 days of age were injected intraperitoneally with 200 mg/kg of alloxan monohydrate after a 16 h fast. The most severe diabetic symptoms occurred in rats injected on day 6.

Keikkila and coworkers (1974) reported the prevention of alloxan-induced diabetes by ethanol administration in mice.

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K.1.3

Streptozotocin-Induced Diabetes

PURPOSE AND RATIONALE

Rakieten and coworkers (1963) reported the diabetogenic activity of the antibiotic streptozotocin. The compound turned out to be specifically cytotoxic to beta-cells of the pancreas.

PROCEDURE

Male Wistar rats weighing 150–220 g fed with a standard diet are injected with 60 mg/kg streptozotocin (Calbiochem) intravenously. As with alloxan, three phases of blood glucose changes are observed. Initially, blood glucose is increased, reaching values of 150–200 mg% after 3 h. Six–eight h after streptozotocin, the serum insulin values are increased up to 4 times, resulting in a hypoglycemic phase which is followed by persistent hyperglycemia. Severity and onset of diabetic symptoms depend on the dose of streptozotocin. After the dose of 60 mg/kg i.v., symptoms occur already after 24–48 h with hyperglycemia up to 800 mg%, glucosuria and ketonemia. Histologi-

cally, the beta-cells are degranulated or even necrotic. A steady state is reached after 10–14 days allowing to use the animals for pharmacological tests.

CRITICAL ASSESSMENT OF THE METHOD

Streptozotocin induced diabetes in laboratory animals, mostly in rats, has become a valuable tool in diabetes research being used by many investigators.

MODIFICATIONS OF THE METHOD

A survey on susceptibility of various species to streptozotocin was given by Frerichs and Creutzfeldt (1971). Multiple low doses of streptozotocin induce immune pancreatic insulinitis in rats thereby mimicking immune type 1 diabetes in humans (Like and Rossini 1976, Rossini et al. 1977). Miller (1990) described the effect of streptozotocin on the golden Syrian hamster using a single i.p injection of 50 mg/kg streptozotocin. Enhancement of streptozotocin induced diabetes in CD-1 mice by cyclosporin A was reported by Iwakiri and coworkers (1987). Grussner and coworkers (1993) induced long-lasting diabetes mellitus in Yorkshire Landrace pigs with a dosage of 150 mg/kg streptozotocin.

Stosic-Grujicic and coworkers (1999) described protection of mice from multiple low dose streptozotocin-induced insulinitis and diabetes by the immunosuppressive drug leflunomide. Bleich and coworkers (1999) found that elimination of leukocyte 12-lipoxygenase in mice ameliorates low dose streptozotocin-induced diabetes by increasing islet resistance to cytokines and decreasing macrophage production of nitric oxide. Masutani and coworkers (1998) studied the role of poly(ADP-ribose)-polymerase (Parp) in streptozotocin-induced diabetes. Parp-deficient (*Parp*^{-/-}) mice were established by disrupting *Parp* exon 1 using the homologous recombination technique. These mice were almost resistant to streptozotocin-induced diabetes.

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K.1.4

Other Diabetogenic Compounds

PURPOSE AND RATIONALE

Several other compounds have been found to induce symptoms of diabetes and/or obesity, such as dithizone (Maske and Weinges 1957, Frerichs and Creutzfeldt 1971, Hansen et al. 1989, Goldberg et al. 1991) or gold-thioglucose (Stauffer et al. 1967, Caterson et al. 1988, Silva and Hernandez 1989, Heydrick et al. 1995) or monosodium glutamate (Sartin et al. 1985).

PROCEDURE

Goldberg and coworkers (1991) injected various chelators, such as dithizone, 8-(p-toluene-sulfonylamino)-quinoline (8-TSQ), and 8-(benzenesulfonylamino)-quinoline (8-BSQ) in a single i.v. dose of 40–100 mg/kg to cats, rabbits, golden hamsters and mice. Dithizone injection causes a triphasic glycemic reaction in rabbits. A phase of initial hyperglycemia is detected after 2 h, followed by a normoglycemic phase after 8 h and a secondary permanent hyperglycemic phase after 24–72 h. Histologically, complete and partial degranulation of beta cells is observed.

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K.1.5

Growth Hormone-Induced Diabetes

Cotes and coworkers (1949) described the diabetogenic action of pure anterior pituitary growth hormone in cats. In intact adult dogs and cats the repeated administration of growth hormone induces an intensively diabetic condition with all symptoms of diabetes including severe ketonuria and ketonemia. Rats of any age subjected to a similar treatment do not become diabetic but grow faster (Young 1945) and show striking hypertrophy of the pancreatic islets.

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K.1.6

Corticosteroid-Induced Diabetes

Ingle (1941) described hyperglycemia and glucosuria in forced fed rats treated with cortisone. In the guinea pig and in the rabbit, experimental corticoid diabetes could be obtained without forced feeding (Hausberger and Ramsay 1953, Abelow and Paschkis 1954). In the rat, the adrenal cortex, stimulated by corticotrophin, has the capacity to secrete amounts of steroids which induce steroid diabetes (Ingle et al. 1946).

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K.1.7

Insulin Deficiency Due to Insulin Antibodies

PURPOSE AND RATIONALE

A transient diabetic syndrome can be induced by injection of guinea pig anti-insulin serum in various species (Moloney and Coval 1955, Wright 1968).

PROCEDURE

Bovine insulin, dissolved in acidified water (pH 3.0), is incorporated in a water-oil emulsion based on complete Freund's adjuvant or a mixture of paraffin oil and lanolin. A dose of 1 mg insulin is injected in divided doses subcutaneously to male guinea pigs weighing 300–400 g. Injections are given at monthly intervals and the guinea pigs are bled by cardiac puncture two weeks after the second and subsequent doses of antigen. It is possible to get 10 ml blood from every animal once a month.

Intravenous injection of 0.25–1.0 ml guinea pig anti-insulin serum to rats induces a dose-dependent

increase of blood glucose reaching values up to 300 mg%. This effect is unique to guinea pig anti-insulin serum and is due to neutralization by insulin antibodies of endogenous insulin secreted by the injected animal. In this way a state of insulin deficiency is induced. It persists as long as antibodies capable of reacting with insulin remain in the circulation. Slow rate intravenous infusion or intraperitoneal injection prolongs the effect for more than a few hours. However, large doses and prolonged administration accompanied by ketonemia, ketonuria, glucosuria, and acidosis are fatal to the animals. After lower doses, the diabetic syndrome is reversible after a few hours.

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K.1.8

Virus-Induced Diabetes

Juvenile-onset (type I) diabetes mellitus may be due to virus infections and β -cell specific autoimmunity (Craighead 1978). The D-variant of encephalomyocarditis virus (EMC-D) selectively infects and destroys pancreatic β -cells in susceptible mouse strains similar to human insulin-dependent diabetes (Yoon et al. 1980, Giron and Patterson 1982, Giron et al. 1983, Vialettes et al. 1983). Adult, male ICR Swiss mice are susceptible to the diabetogenic effect of the D-variant of encephalomyocarditis virus in contrast to adult C3H/HeJ male mice which are relatively resistant. Pretreatment with cyclosporin A, a potent immunosuppressive drug, results in increased severity and incidence of diabetes in susceptible ICR Swiss mice and induction of diabetes in resistant C3H/HeJ mice (Gould et al. 1985).

MODIFICATIONS OF THE METHOD

Hirasawa and coworkers (1997) studied the possible role of macrophage-derived soluble mediators in the pathogenesis of encephalomyelitis virus-induced diabetes in mice. The inactivation of macrophages prior to viral infection resulted in the prevention of diabetes. Utsugi and coworkers (1992) demonstrated that intraperitoneal inoculation with NDK25, a variant of encephalomyocarditis virus which has been cloned from

the M variant of encephalomyocarditis virus, caused DBA/2 mice to develop non-insulin-dependent diabetes mellitus. See and Tilles (1995) challenged CD-1 mice with a diabetogenic strain (E2) of coxsackievirus B4. Islet cell destruction was associated with chronic islet cell inflammation, elevation of islet cell antibody, and prolonged presence of viral RNA in the pancreas. Stubbs and coworkers (1994) investigated the effect of Kilham rat virus (KRV) infection on GLUT2 expression in diabetes-resistant BB/Wor rats. Viral antibody-free diabetes resistant rats did not develop spontaneous diabetes, but inoculation with Kilham rat virus induced autoimmune beta cell-destruction and hyperglycemia. Hayashi and coworkers (1995) investigated the role of adhesion molecules in the reovirus type2-induced diabetes-like syndrome in mice. Ellerman and coworkers (1996) studied Kilham rat virus induced autoimmune diabetes in multiple strains of rat.

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K.2 Genetically Diabetic Animals

GENERAL CONSIDERATIONS

Several animal species, mostly rodents, were described to exhibit spontaneously diabetes mellitus on a hereditary basis. These findings were highly appreciated with the expectation to get more insight into the pathogenesis of diabetes in humans. During the last few years since the discovery of leptin (Zhang et al. 1994) and its downstream signal transduction cascade (Friedman and Halaas 1998) tremendous new insight of the genetics of diabetic and obese animal disease models derived. Up to now at least 6 genetically diabetic animal models exhibit defects in the leptin pathway: *Theob* mutation in the mouse resulted in leptin deficiency. *The^{db}* mutation in the mouse and the *cp* and *fa* mutations in the rat are different mutations of the leptin receptor gene. The *fat* mutation in the mouse results in a biologically inactive carboxipeptidase E, which processes the prohormone conversion of POMC into α -MSH, which activates the hypothalamic MC4 receptor. Finally the Agouti yellow (*y*) mouse exhibit an ubiquitous expression of the Agouti protein which represents an antagonist of the hypothalamic MC4 receptor.

Symptoms of diabetes and obesity are overlapping in many animal models (see also L.2. Genetically obese animals).

CRITICAL ASSESSMENT

The pathophysiological mechanisms which finally lead to the diabetes phenotype (hyperglycemia, hyperinsulinemia and insulin resistance) exhibited of the various animal disease models for non-insulin dependent diabetes do not necessarily be identical to those in human disease. Therefore, detailed knowledge about the (patho-)physiology of these animal disease models is a prerequisite for interpretation of experimental results and their value for the human disease.

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K.2.1

Spontaneously Diabetic Rats

The occurrence of spontaneous diabetes has been reported in several strains of rats.

BB Rat

The BB rat (Bio Breeding (BB) rat) is a model of spontaneous diabetes associated with insulin deficiency and insulinitis due to autoimmune destruction of pancreatic beta cells. (Nakhoda et al. 1977 and 1978, Like et al. 1982, Oschilewski et al. 1985, Lee et al. 1988, Solomon et al. 1989, Papaccio and Mezzogiorno 1989, Kolb et al. 1990, Velasquez et al. 1990, Lefkowitz et al. 1990, Gottlieb et al. 1990, Ellerman et al. 1993). Diabetes is inherited as an autosomal recessive trait and develops with equal frequency and severity among males and females. The onset of clinical diabetes is sudden, and occurs at about 60–120 days of age. Within several days, diabetic animals are severely hyperglycemic, hypoinsulinemic, and ketotic unless insulin treatment is instituted. Pipeleers and coworkers (1991) described the transplantation of purified islet cells in diabetic BB rats. Hao and coworkers (1993) reported that the immunosuppressive agent mycophenolate mofetil can prevent the development of diabetes in BB rats. Klötting and Vogt (1991) characterized the features of a subline of diabetes-prone BB rats (BB/OK rats). The circadian variations in blood pressure and heart rate of this strain were compared with spontaneously hypertensive rats (Berg et al. 1997).

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WBN/Kob RAT

Spontaneous hyperglycemia, glucosuria and glucose intolerance have been observed in aged males of an inbred Wistar strain, named the WBN/Kob rat (Nakama et al. 1985, Tsuchitani et al. 1985, Koizumi 1989, Shimoda et al. 1993). These animals exhibit impaired glucose tolerance and glucosuria at 21 weeks of age. Obvious decreases in the number and size of islets are found already after 12 weeks of age. Fibrinous exudation and degeneration of pancreatic tissue are observed in the exocrine part, mainly around degenerated islets and pancreatic ducts in 16 weeks old males. These rats develop demyelinating, predominantly motor neuropathy, later accompanied by axonal changes (Yagihashi et al. 1993).

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Cohen Diabetic Rat

Diabetes in Cohen rats is characterized by hyperglycemia, glucosuria, and hyperinsulinemia, with late development of hypoinsulinemia, insulin resistance, and a decrease in the number and sensitivity of insulin receptors. The rats develop overt diabetes and diabetes related complications when fed a diet rich in sucrose or other refined sugars and poor in copper content, but not when fed a starch or stock diet (Cohen 1972, Velasquez et al. 1990).

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Goto–Kakizaki Rat

Non-obese, insulin-resistant Goto–Kakizaki (GK) rats are a highly inbred strain of Wistar rats that spontaneously developed type II diabetes. Defects in glucose-stimulated insulin secretion, peripheral insulin resistance, and hyperinsulinemia are seen as early as 2 to 4 weeks after birth. Impaired skeletal muscle glycogen synthase activation by insulin was observed, accompanied by chronic activation of diacylglycerol-sensitive protein kinase C.

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Zucker-Fatty Rat

The Zucker-fatty rat is a classic model of hyperinsulinemic obesity. (Zucker 1965). Obesity is due to a simple autosomal recessive (*fa*) gene and develops at an early age. Obese Zucker rats manifest mild glucose intolerance, hyperinsulinemia, and peripheral insulin resistance similar to human type 2 diabetes. However, their blood sugar level is usually normal throughout life (Bray 1977, Clark et al. 1983, McCaleb and Sredy 1992, Abadie et al. 1993, Alamzadeh et al. 1993, Kasim et al. 1993, Galante et al. 1994).

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Zucker Diabetic Fatty Rat (*Zdf/Drt-Fa*)

The obese Zucker Diabetic Fatty rat originally derived from the Zucker fatty rat (Peterson et al. 1990), and has the identical mutation (*fa*) in the leptin receptor. In addition a defect in the insulin promoter has been described (Griffen et al. 2001). Insulin promoter activity is reduced 30–50% in homozygous ZDF fetal islets, and insulin mRNA levels are similarly reduced by 45%. Only the males become diabetic at the age of around 10 weeks spontaneously with hyperglycemia of about 20 mmol/l; the females develop diabetes only when feeding a high-fat diet. Diabetes phenotype develops due to lipotoxicity to the β -cell (Lee et al. 1994). These rats are characterized besides hyperglycemia by insulin resistance, moderate hyperinsulinemia, extreme hyperphagia due to the loss of calories by glucosuria and obesity.

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Wdf/Ta-Fa Rat

The WDF/Ta-*fa* rat, commonly referred to as the Wistar fatty rat, is a genetically obese, hyperglycemic rat established by the transfer of the fatty (*fa*) gene from the Zucker rat to the Wistar Kyoto rat. (Ikeda et al. 1981, Kava et al. 1989, Velasquez et al. 1990). The Wistar fatty rat exhibits obesity, hyperinsulinemia, glucose intolerance, hyperlipidemia, and hyperphagia similar to Zucker rats being, however, more glucose intolerant and insulin resistant than Zucker rats. Hyperglycemia is usually not observed in females but can be induced by addition of sucrose to the diet. Kobayashi and coworkers (1992) found an increase of insulin sensitivity by activation of insulin receptor kinase by Pioglitazone in Wistar fatty rats (*fa/fa*). Sugiyama and

coworkers (1992) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

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OLETF Rat

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long-Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and named OLETF. The characteristic features of OLETF rats are: (1) late onset of hyperglycemia (after 18 weeks of age), (2) a chronic course of disease, (3) mild obesity, (4) inheritance by males, (5) hyperplastic foci of pancreatic islets, and (6) renal complications (nodular lesions). The clinical and pathological features of disease in OLETF rats resemble those of human NIDDM. Administration of diazoxide (0.2% in diet), an inhibitor of insulin secretion, to OLETF rats from the age of 4 to 12 weeks completely prevented the development of obesity and insulin resistance (Aizawa et al. 1995). Ishida and coworkers (1995) found that insulin resistance preceded impaired insulin secretion in OLETF rats.

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ESS-Rat

The occurrence of spontaneous diabetes in a colony of rats (Stilman Saldago), called eSS-rat was reported by Tarrés and coworkers (1981). The animals show abnormal glucose tolerance tests from the age of 2 months onwards. The syndrome consists of a mild type of diabetes that does not diminish the longevity of the animals. Six-months old rats show disruption of the islet architecture and fibrosis of the stroma (Dumm et al. 1990).

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Obese SHR Rat

The strain of obese SHR rats was developed by Koletsky (1973, 1975) by mating a spontaneous hypertensive female rat of the Kyoto-Wistar strain with a normotensive Sprague Dawley male. After several generations of selective inbreeding, these obese SHR exhibited obesity, hypertension, and hyperlipidemia. In addition, some rats developed hyperglycemia and glucosuria associated with giant hyperplasia of pancreatic islets. From these rats, several substrains were developed, such as the JCR:LA-corpulent rat which exhibits a syndrome characterized by obesity, hypertriglyceridemia and hyperinsulinemia with impaired glucose tolerance (Russell et al. 1994). Reduced insulin receptor signaling was found in the obese spontaneously hypertensive Koletsky rat (Friedman et al. 1997).

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SHR/N-cp Rat

The congenic SHR/N-cp rat strain, developed at the National Institutes of Health, USA, (Hansen 1983, Michaelis et al. 1986, Hansen 1988, Adamo et al. 1989) was derived by mating a male Koletsky rat heterozygous for the corpulent gene (cp/+) to a female rat of the Okamoto strain. After a minimum of 12 backcrosses, homozygous (cp/cp) SHR/N-cp rats exhibit obesity, mild hypertension, hyperinsulinemia, and glucose intolerance.

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BHE Rat

The BHE rat colony was originally developed by breeding black and white hooded rats of the Pennsylvania State College strain and albino rats of the Yale (Osborne Mendell) strain. The BHE rat is a model in

which the diabetic state is manifested only at maturity. BHE rats have hyperinsulinemia at 50 days of age associated with glucose intolerance and tissue resistance to insulin. Later on, BHE rats have less hyperinsulinemia with reduced pancreatic insulin stores, and show mild hyperglycemia and hyperlipidemia.

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LEW.1AR1/Ztm-iddm RAT

Lenzen et al. (2001), Jörns et al. (2004) described a Type I (insulin-dependent) diabetes mellitus rat model (LEW.1AR1/Ztm-iddm), which arose through a spontaneous mutation in a congenic Lewis rat strain with a defined MHC haplotype (RT1.Aa B/Du Cu). Diabetes appeared in the rats with an incidence of 20% without major sex preference at 58+/-2 days. The disease was characterised by hyperglycaemia, glycosuria, ketonuria and polyuria. In peripheral blood, the proportion of T lymphocytes was in the normal range expressing the RT6.1 differentiation antigen. Islets were heavily infiltrated with B and T lymphocytes, macrophages and NK cells with beta cells rapidly destroyed through apoptosis in areas of insulinitis.

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K.2.2

Spontaneously Diabetic Mice

KK Mouse

Nakamura (1962, 1967) reported on a diabetic strain of the KK-mouse. The animals were moderately obese and showed polyphagia and polyuria. Mice at the age of seven months or older showed glucosuria and blood sugar levels up to 320 mg%. The pancreatic insulin content was increased, but histologically degranulation of the β -cells and hypertrophy of the islets were found.

Sections of the liver showed a reduction of glycogen and an increase in lipid content.

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KK-A^Y Mouse

Iwatsuka and coworkers (1970) reported on yellow KK mice (also named KK-A^Y mice), carrying the yellow obese gene (A^Y). These mice develop marked adiposity and diabetic symptoms in comparison with their littermates, black KK mice. Blood glucose and circulating insulin levels as well as HbA_{1c} levels were increased progressively from 5 weeks of age. Degranulation and glycogen infiltration of B cells were followed by hypertrophy and central cavitation of islets. Lipogenesis by liver and adipose tissue were increased. Insulin sensitivity of adipose tissue was more remarkably reduced than in black KK mice to its complete loss at 16 weeks of age. Renal involvement is uniquely marked by early onset and rapid development of glomerular basement membrane thickening (Diani et al. 1987).

KK-A^Y mice can be used to demonstrate the extrapancreatic action of antidiabetic drugs, such as glimepiride, a novel sulfonylurea (Satoh et al. 1994). Sohda and coworkers (1990) evaluated ciglitazone and a series of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazolidinediones for hypoglycemic and hypolipemic activities in yellow KK mice. Hofmann and coworkers (1992) evaluated the expression of the liver glucose transporter GLUT2 and the activity and the expression of phosphoenolpyruvate carboxykinase in the liver of obese KKA^Y mice after treatment with the oral antidiabetic agent pioglitazone.

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NOD Mouse

The NOD mouse strain was established by inbreeding diabetic CTS mice derived originally from the JCL-ICR strain. Like the BB rat, the NOD mouse is a model of insulin dependent diabetes mellitus and develops hypoinsulinemia secondary to autoimmune destruction of pancreatic β cells in association with insulinitis and auto antibody production. Following insulinitis, destruction of the insulin-producing pancreatic β cells ensues in many (but not all) mice, with physiologic manifestations of insulin depletion appearing between 3 and 7 months of age. The onset of diabetes can be prevented by an immunomodulating drug (Baeder et al. 1992) or by a soluble interleukin-1 receptor (Nicoletti et al. 1994). Hutchings and Cooke (1995) compared the protective effects afforded by intravenous administration of bovine or ovine insulin to young NOD mice. Bergerot and coworkers (1997) reported that feeding small amounts (2–20 μ g) of human insulin conjugated to cholera toxin B subunit can effectively suppress β -cell destruction and clinical diabetes in adult NOD mice. Insulin-dependent diabetes mellitus in NOD Mice is the result of a CD4⁺ and CD8⁺ T cell-dependent auto-immune process directed against the pancreatic β -cells (Serreze and Leiter 1994, Verdaguer et al. 1997).

Elias et al. (1990) described induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein.

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Obese Hyperglycemic Mice

Ingalls and coworkers (1950), Mayer and coworkers (1951), Bleisch and coworkers (1952) observed hereditary diabetes in genetically obese mice. The obese hyperglycemic mice were glycosuric, the non-fasting blood sugar levels were about 300 mg%, but neither ketonuria nor coma were observed. One of the most interesting features was insulin-resistance; doses as high as 400 IU/kg had little effect on blood sugar. The serum insulin-like activity was high, the islands of Langerhans were hypertrophic, their insulin content was increased and the liver glycogen stores were decreased. Kidneys and other organs did not show pathological changes. Obviously, the diabetic condition of this and other strains of obese hyperglycemic mice is different from that of the human diabetic patient. The *ob* mutation was identified as a mutation in the leptin gene in adipose tissue (Zhang et al. 1994) and the substitution of leptin reverses the obese and diabetic phenotype completely (Halaas et al. 1995). Other strains or substrains of mice with obesity and hyper-

glycemia have been described by Dickie (1962), Westman (1968), Stein and coworkers (1970), Coleman and Hummel (1973), and Herberg and Coleman (1977). Gill and Yen (1991) studied the effect of ciglitazone on endogenous plasma islet amyloid polypeptide (amylin) and insulin sensitivity in obese-diabetic viable yellow mice (VY/Wfl-A^{vy/a}).

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Diabetic db/db Mice

The diabetes *db/db* mouse strain is derived from an autosomal recessive mutation having occurred spontaneously in mice of the C57BL/KsJ strain which was identified as a mutation in the leptin receptor gene (Tartaglia et al. 1995). On this basis, the diabetes mouse (C57BL/6J *db/db*) consistently develops a severe diabetic syndrome similar to that found in the C57BL/KsJ *ob/ob* mouse, characterized by early onset of hyperinsulinemia, and hyperglycemia up to

20 to 25 mmol/l. (Hummel et al. 1966, Coleman and Hummel 1967, Like et al. 1972). The *db/db* mouse, in contrast to the *ob/ob* mouse, develops significant nephropathy (Gardner 1978). Mutations on the leptin receptor result in an obese phenotype identical to that of *ob* mice (Li et al. 1998). C57BL/KsJ *ob/ob* mice are phenotypically the same as other strains of *db* mice. The leptin receptor (Ob-R) gene encodes 5 alternatively spliced forms, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd (Lee et al. 1996). In the *db/db* mouse strain, the Ob-Rb transcript contains an insert with a premature stop codon as a result of abnormal splicing (Chen et al. 1996, Friedman and Halaas 1998).

Coleman and Hummel (1969) joined adult diabetic mice (*db/db*) of the C57BL/Ks strain in parabiosis with normal mice of the same sex. Little, if any, amelioration of the disease was observed in parabiont diabetics and no symptoms of diabetes were observed in the normal parabiont. Instead, the normal partners lost weight, became hypoglycemic, and died of apparent starvation 50 days after surgery. In contrast, the diabetic partners gained weight rapidly and remained diabetic. Raizada and coworkers (1980) demonstrated a decrease of insulin receptors and impaired responses to insulin in fibroblastic cultures from the diabetic *db/db* mouse.

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K.2.3 Chinese Hamster

Diabetes Obesity Syndrome in CBA/Ca mice

A spontaneous maturity onset diabetes obesity syndrome occurs in a small proportion (10–20%) of male CBA/Ca mice. Inbreeding can increase the incidence to 80%. It occurs at 12–16 weeks of age, and is characterized by hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and an impaired glucose tolerance. The mice are also resistant to exogenous insulin. Female mice remain normal except for a slight increase in serum insulin. The male obese diabetic mice have a normal life expectancy.

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Wellesley Mouse

The Wellesley mouse, described first by Jones (1964), is a hybrid with predisposition to diabetes mellitus. The diabetic animals have elevated levels of immunoreactive insulin in serum, enlarged pancreatic islets and reduced insulin responsiveness in peripheral tissues (Cahill et al. 1967, Gleason et al. 1967, Like and Jones 1967).

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Meier and Yerganian (1959, 1961) described the occurrence of hereditary diabetes mellitus in the Chinese hamster (*Cricetulus griseus*). Blood sugar levels of diabetic hamsters were elevated from a normal of 110 mg% up to 600 mg%. Severe polyuria, glycosuria, ketonuria, and proteinuria were observed. The diabetic condition could be improved by administration of insulin, and oral antidiabetic drugs were effective in mildly diabetic hamsters. Pathological changes were seen in histological sections of pancreas, liver and kidney. The number of pancreatic islets was decreased, and the cells of the remaining islets were abnormal. This animal model has been studied by several authors.

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K.2.4

Other Species with Inherited Diabetic Symptoms

SAND Rat

The sand rat (*Psammomys obesus*) lives in the desert regions of North Africa and the Near East. In the laboratory the animals develop diabetic symptoms when fed Purina laboratory chow instead of an all vegetable diet (Hackel et al. 1965 and 1967, Miki et al. 1967, deFronzo et al. 1967, Brodoff et al. 1967, Strasser 1968). The diabetic syndrome in the sand rat usually develops within 2–3 months with variations in severity between the animals. Severely hyperglycemic animals die prematurely from ketosis. Initially, the pancreatic islets appear normal. In the intermediate stage of the disease, degranulation of pancreatic β cells is observed. This is followed by β cell degeneration and necrosis with resultant insulinopenia and ketonuria. Histological studies by Dubault and coworkers (1995) showed insulinitis in animals who became insulin-dependent in later stages and recommended *Psammomys obesus* as a model of latent progression to insulin-deficiency in type 2 diabetes patients.

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Spiny Mouse

The spiny mouse (*Acomys cahirinus*) is a small rodent living in the semi-desert areas of the Eastern Mediterranean (Pictet et al. 1967). Diabetes occurs in about 15% of the animals under laboratory conditions accompanied by hyperplasia of the endocrine pancreas. Great variations in the appearance and severity of diabetes and obesity occur in this species. Some animals show obesity, mild hyperglycemia, and hyperinsulinemia. Others have frank hyperglycemia with glucosuria that leads to fatal ketosis. Regardless of the stage of the disease, all spiny mice characteristically have massive hyperplasia of pancreatic islets and increased pancreatic insulin content. Despite the large insulin stores, plasma insulin response to glucose is delayed or impaired suggesting an impairment of the insulin release mechanism.

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African Hamster (*Mystromys albicaudatus*)

Spontaneous diabetes mellitus was described in South African hamsters (*Mystromys albicaudatus*) by Packer and coworkers (1970), Stuhlman and coworkers (1972, 1974, 1975), Schmidt and coworkers (1974). Characteristics established as part of the diabetic syndrome in this species include hyperglycemia, glucosuria, ketonuria, polyuria, polyphagia and polydipsia. Pancreatic lesions include β -cell vacuolization, glycogen infiltration, nuclear pycnosis, margination of organelles, and β -cell death.

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Tuco-Tuco

The diabetic syndrome in Tuco-tucos (*Ctenomys talarum*) is similar to that in sand rats and spiny mice (Wise et al. 1972). However, Tuco-tucos tend to have less hyperglycemia and are less prone to ketosis. Many animals, mainly males, become hyperphagic and quite obese. Degranulation of β cell is the usual lesion in the pancreas, but amyloid hyalinization of islets has been observed in a few animals.

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Macaca Nigra

A high incidence of spontaneous diabetes mellitus was found in *Macaca nigra* (Celebes black apes) with analogies to human diabetes (Howard 1972, 1974a, b, 1975). Abnormal signs include hyperglycemia, decreased clearance of glucose, in intravenous tolerance tests, reduced insulin secretion and increased serum lipids. Insulin secretory capacity is lost concomitant with amyloid infiltration into the islets of Langerhans. Secondary manifestations are atherosclerosis, thickened basement membranes of muscle capillaries, and cataracts. The genetic predisposition in these monkeys is exacerbated by changes in diet and environment.

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K.2.5

Transgenic Animals and Knockout Mice

GENERAL CONSIDERATIONS

Insulin exerts its diverse biological effects by binding to and activating the membrane-bound insulin receptor (IR), thereby initiating the insulin signal transduction cascade (see K.6.3 and K.6.3.2). IRs are not only present in the classical insulin target tissues, i. e., adipose tissue, skeletal muscle, and liver, but are also widely expressed throughout the organism in tissues such as pancreas, the central nervous system (CNS), lymphatic cells, and kidney. Signals initiated by the activated IR result in the diverse biological effects of insulin, such as the stimulation of glucose transport, the inhibition of gluconeogenesis, the stimulation of protein and lipid synthesis, and the regulation of gene transcription. Because the roles of many components of the signaling pathway were initially characterized *in vitro*, more recent research has focused on the analysis of insulin action *in vivo* by generating mice with targeted disruption of genes for these components. Since the pattern of inheritance of type II diabetes points to a disease of polygenic nature, not only single gene-deficient mice but also models with combined genetic

disruptions have been created in an attempt to represent the polygenic nature of the disease.

Realization that metabolic diseases do not only involve many genes but also many organs necessitated engineering the mouse genome with spatial and temporal controls. This requires the generation of premutant mice whereby the alleles of interest is flanked by recognition sites for DNA recombinases such as Cre (loxP sites) or Flp (Frt sites; Branda and Dymecki 2004). When such premutant mice are bred with transgenic mice that express the corresponding DNA recombinase in a tissue-specific fashion, the gene of interest is inactivated only in that particular tissue. An added sophistication of spatially controlled mutagenesis is the inclusion of temporal control, which is achieved by using ligand-activated chimeric recombinases composed of fusion of the recombinase with the ligand binding domain of a nuclear receptor (Metzger and Chambon 2001). Employing spatially and temporally controlled mutagenesis revealed that metabolic perturbation in one tissue encroaches on the metabolism of another. This tissue communication and crosstalk allows for metabolic flexibility, with respect to the use or production of metabolic fuel. Understanding this may ultimately reveal the genetic determinants that limit the degree of metabolic flexibility, thus promoting metabolic diseases in humans (Bickel 2004).

The first knockout models of insulin resistance aimed at the disruption of major molecules in IR signaling in each tissue. The successful generation of viable, heterozygous IR knockout mice showed that 50% of IR expression is sufficient for the maintenance of physiological blood glucose concentrations. In contrast, homozygous IR-deficient mice rapidly develop diabetic ketoacidosis and die within 3–7 days after birth (Accili et al. 1996, Joshi et al. 1996), showing the indispensability of IR for the control of glucose metabolism. The new techniques for conditional gene inactivation in mice are particularly helpful if conventional knockout of a gene results in embryonic lethality, preventing the analysis of gene function in the adult. Conditional mutagenesis enables the inactivation of a gene of interest in a specific tissue or in a temporally controlled manner, thereby providing new insights into the contribution of a gene to a complex physiological phenotype such as insulin resistance.

Skeletal Muscle and Insulin Resistance

Because insulin resistance in skeletal muscle is one of the earliest detectable defects in type II diabetic patients, muscle-specific insulin receptor knockout (MIRKO) mice have been generated as a model for

muscle-specific insulin resistance. MIRKO mice displayed elevated fat mass, serum triglycerides, and free fatty acids, indicating that insulin resistance in muscle contributes to the altered fat metabolism associated with type II diabetes (Brüning et al. 1998). Additionally, Kim and coworkers (2001) demonstrated that IR deficiency in muscle promotes redistribution of substrates to adipose tissue, thereby contributing to increased adiposity in MIRKO mice. Surprisingly, blood glucose, serum insulin, and glucose tolerance were normal in these mice, initially leading to the interpretation that tissues other than muscle are more essential for insulin-regulated glucose disposal than previously assumed. However, Zisman and coworkers (2000) disrupted the gene for the glucose transporter 4 (GLUT4), which mediates glucose transport in response to insulin, selectively in mouse muscle tissue. They found that Glut4-mediated glucose uptake in muscle was indeed essential for the maintenance of normal glucose homeostasis. Consistently, mice with a combined muscle-specific functional inactivation of IR and the closely related insulin-like growth factor receptor (IGF-1R) also display a complete type II diabetic phenotype, implicating a compensatory role of IGF-1R for mediating insulin's stimulation of glucose transport *via* Glut4 in muscle (Fernandez et al. 2001).

Adipose Tissue and Insulin Resistance

The strong association between the world-wide epidemic of obesity and dramatically increasing prevalence of insulin resistance and type II diabetes has prompted recent research to focus on the mechanisms linking adipose tissue to whole body insulin sensitivity, β -cell function, and overall glucose metabolism. Newborn mice with IR deficiency show a marked reduction in white adipose tissue mass, pointing to a role for IR in the regulation of adipocyte growth and differentiation (Accili et al. 1996). Mice with an adipose tissue-specific knockout of the IR (FIRKO) also display a reduced fat mass, loss of the normal correlation of plasma leptin and body weight, and protection against both obesity and obesity-related glucose intolerance. Interestingly, they also exhibit an 18% extended lifespan (Bluher et al. 2003). Strikingly, whereas adipocytes of control mice exhibit a bell-shaped size distribution, adipocytes of FIRKO mice demerge into groups of small and large cells. Interestingly, these differently sized cells also show different expression of fatty acid synthase and the transcription factors CCAAT enhancer binding protein (C/EBP)- α as well as sterol regulatory element-binding protein 1 (SREBP1). Thus inactivation of the IR revealed a pre-

viously unrecognized heterogeneity of adipose tissue. Consistent with the phenotype of GLUT4-null mice, which display a depletion of fat stores (Katz et al. 1995), the selective disruption of GLUT4 in adipose tissue caused an impaired glucose transport, followed by development of insulin resistance. The role of adipose tissue in muscle-specific IR deficiency was addressed in mice with a targeted inactivation of IR-mediated signaling in both muscle and adipose tissue (Lauro et al. 1998). These mice did not become diabetic despite peripheral insulin resistance and a mild impairment of β -cell function. Together, these data suggest that there may be a critical threshold in whole body insulin resistance that finally leads to a diabetic phenotype. Moreover, this insulin resistance might result in excessive demands on the pancreatic β -cells to secrete insulin, leading to consecutive decompensation of these cells. These data clearly assign adipocytes an important role in lipid storage, development of obesity, and regulation of glucose homeostasis.

Role of the β -Cell in Impaired Glucose Tolerance

Mice with a specific disruption of the IR gene in β -cells show a selective loss of insulin secretion in response to glucose and a progressive impairment of glucose tolerance (Kulkarni et al. 1999), indicating that insulin stimulates its own secretion via IRs on β -cells, thus playing an important functional role in glucose sensing by the pancreatic β -cell. Therefore, defects in insulin signaling at the level of the β -cell may contribute to the observed alterations in insulin secretion in type 2 diabetes. Interestingly, a β -cell-specific IGF-1R knockout model also underscored a role for this receptor in the control of glucose-stimulated insulin secretion and glucose tolerance (Kulkarni et al. 2002). The interplay between insulin resistance and insulin secretory defects has also been addressed by the generation of mice deficient for insulin receptor substrate 1 (IRS-1) and β -cell glucokinase (GK). The heterozygous β -cell GK knockout is characterized by decreased insulin secretion in response to glucose, whereas IRS-1-deficient mice are insulin resistant but do not develop overt diabetes (Abe et al. 1998). In contrast to either individual mutation, the double-knockout mice developed a diabetic phenotype (Terauchi et al. 1997), demonstrating that the combination of individual minor defects in insulin action or insulin secretion can cause overt diabetes.

Insulin Resistance in the Brain

The liver-specific insulin receptor knockout (LIRKO) mouse, as expected, exhibits a dramatic phenotype

with severe insulin resistance and progressive liver failure (Michael et al. 2000), mirroring the critical role of insulin signaling in liver for regulating glucose homeostasis and maintaining normal hepatic function. However, IRs and insulin-signaling proteins are not exclusively expressed in classic insulin target tissues; they are also widely distributed throughout the CNS. CNS-specific disruption of the IR gene (NIRKO) revealed an important role of IRs in the regulation of energy disposal, fuel metabolism, and reproduction (Brüning et al. 2000). It has very recently been shown in conventional IR-deficient mice that combined restoration of IR function selectively in brain, liver, and pancreatic β -cells rescues these mice from neonatal death, prevents diabetes in a majority of animals, and restores adipose tissue content, lifespan, and reproductive function. Interestingly, IR substitution either limited to brain or liver and pancreatic β -cells was sufficient to prevent neonatal death but not lipotrophic diabetes, leading to the surprising finding that IR signaling in nontypical insulin target tissues like the brain seems to be crucial to maintain fuel homeostasis and prevent diabetes.

Inactivation of IRS Proteins

Aside from IR knockouts, various molecules of the signal transduction pathway initiated by IR have been disrupted, such as IRS-1, which was believed to be the principal substrate for the IR and IGF-1R. Mice deficient for IRS-1 exhibit impaired glucose tolerance and a decrease in insulin/IGF-1-stimulated glucose uptake *in vivo* and *in vitro*, thereby providing a mouse model of genetically determined insulin resistance (Abe et al. 1998, Jenkins and Storlien 1997). Additionally, elevated blood pressure and plasma triglyceride levels were observed, as well as impaired endothelium-dependent vascular relaxation, indicating that insulin resistance plays an important role in the clustering of coronary risk factors leading to accelerated atherosclerosis. The residual insulin/IGF-1 action in IRS-1-deficient mice correlated with the existence of an alternative tyrosine-phosphorylated protein (IRS-2) (Araki et al. 1994), which is also capable of activating the signaling cascade. Surprisingly, disruption of IRS-2 impaired peripheral insulin signaling and pancreatic β -cell function, resulting in progressive deterioration of glucose homeostasis (Withers et al. 1998). This phenotype results from insulin resistance in liver and skeletal muscle and β -cell dysfunction. Succeeding studies indicated tissue-specific functions for IRS-1 and IRS-2 in mediating the metabolic effects of insulin *in vivo*, IRS-1 having a major role in skeletal

muscle and IRS-2 in liver, muscle, adipose tissue, pancreatic β -cells, and reproductive tissue. Interestingly, mice with a double knockout of IRS-1 and the subsequently identified IRS-3 (Lavan et al. 1997) displayed a phenotype of early-onset severe lipatrophy associated with hyperglycemia, hyperinsulinemia, and decreased plasma levels of the anorexigenic hormone leptin. The IRS-1/IRS-3 double-knockout phenotype in mice (Lausten et al. 2002) might mimic the situation in humans better than the single IRS-1 knockout.

The concept that several subclinical genetic alterations in insulin action can synergize to result in overt diabetes was tested in mice double heterozygous for IR and IRS-1 alleles with a $\sim 50\%$ reduction in expression of either protein (Brüning et al. 1997). In these mice, the combined genetic defects led to aggravation of insulin resistance with 5- to 50-fold elevated plasma insulin levels and respective β -cell hyperplasia, and $\sim 40\%$ of the animals developed overt diabetes at the age of 4–6 mo. This mouse model of type II diabetes, in which diabetes arises in an age-dependent manner from the interaction between two genetically determined, subclinical defects in the insulin-signaling cascade, demonstrates the important role of epistatic interactions in the pathogenesis of common diseases with non-Mendelian genetics. Moreover, this model has been used successfully to identify further modifying C57BL/6 loci regulating insulin sensitivity (Almind et al. 2003).

Inactivation of PPARs

It has been shown that the abnormal accumulation of lipids in tissues other than adipose adversely affects insulin sensitivity, indicating a complex system for the comprehensive control of lipid and glucose homeostasis. The key coordinators in this metabolic axis are members of the nuclear hormone receptor superfamily. Among those, PPARs (see K.6.4.4) respond to small lipid agents, e. g., dietary fatty acids, and contribute a key mechanism in the regulation of lipid and glucose metabolism (reviewed by Rangwala and Lazar 2004). The important physiological role of the PPARs, i. e., PPAR- α , - β/δ and - γ , was deduced from findings identifying the PPARs as primary targets of two key classes of synthetic compounds that have been used in the successful treatment of diabetes and dyslipidemia. In particular, thiazolidinedione (TZD) insulin sensitizers are potent and specific PPAR- γ ligands and activators. Fibrates have predominant activity as PPAR- α agonists, favorably affecting serum lipid levels. Diverse knockout models have been created to study the function of the single PPAR isoforms *in vivo*.

Consistent with the concept that PPAR- α is the member of the PPAR family that mediates cellular lipid utilization, pharmacological inhibition of cellular fatty acid flux in mice lacking PPAR- α caused massive hepatic and cardiac lipid accumulation, hypoglycemia, and death in 100% of male and 25% of female animals, demonstrating a pivotal role for PPAR- α in lipid and glucose homeostasis *in vivo*. Nevertheless, there was no major phenotypic defect detectable in PPAR- α knockouts without pharmacological challenge (Djouadi et al. 1998). Muoio and coworkers (2002) showed that skeletal muscle of PPAR- α -deficient mice exhibited only minor changes in fatty acid homeostasis, and even mRNA expression of known PPAR- α target genes in muscle tissue was not significantly affected. They proposed that this finding might be explained by high levels of PPAR- β/δ compensating for the lack of PPAR- α , suggesting redundancy in the functions of PPARs as transcriptional regulators of fatty acid homeostasis.

In contrast to the embryonic lethality of homozygous PPAR- γ knockouts (Barak et al. 1999), mice deficient only for the PPAR- $\gamma 2$ isoform survived, exhibiting an overall reduction in white adipose tissue, less lipid accumulation, and decreased expression of adipogenic genes in adipose tissue (Zhang et al. 2004). Consistently, embryonic fibroblasts of PPAR- $\gamma 2$ knockouts showed a dramatically reduced capacity for adipogenesis *in vitro*. In addition, insulin sensitivity was impaired in these mice, with decreased expression of IRS-1 and Glut4 in skeletal muscle, but TZDs were able to normalize this insulin resistance (Zhang et al. 2004). In contrast, heterozygous PPAR- γ knockouts show reduced disposition to insulin resistance. Strikingly, this phenotype is blunted by treatment with a synthetic PPAR- γ ligand of the TSD class, indicating that optimal levels of PPAR- γ activity are crucial for its beneficial effects. To address these obvious incongruities, PPAR- γ has been selectively disrupted in liver, adipose tissue, and muscle. Briefly, although adipose tissue appears to be the main site of TZD action, the conditional knockouts highlight important functions for muscle and liver PPAR- γ in the control of body composition and insulin sensitivity. PPAR- γ deficiency in adipose tissue leads to progressive loss of fat, hyperlipidemia, fatty liver, and accompanying hepatic insulin resistance (He et al. 2003). The mice can maintain normal whole body glucose homeostasis and normal insulin sensitivity only as long as some adipose tissue is present. These studies identify a molecular link between blood glucose homeostasis and lipid metabolism, providing a genetic basis for the observed

phenotypic correlation between obesity and type II diabetes mellitus.

Transgenic Animal Model for Type I Diabetes

Aichele and coworkers (1994) used a synthetic peptide corresponding to an immunodominant epitope of lymphocytic choriomeningitis virus glycoprotein (LCMV GP) to prime or to tolerize CD8⁺ T cells *in vivo*. Peptide-specific tolerance was then examined in transgenic mice expressing LCMV GP in the β islet cells of the pancreas. These mice developed CD8⁺ T cell-mediated diabetes within 8–14 days after LCMV infection. Specific peptide-induced tolerance prevented autoimmune destruction of β islet cells and diabetes in this transgenic mouse model. Oldstone and coworkers (1991) showed that virus infection triggers insulin-dependent diabetes mellitus in a transgenic mouse model. Ablation of tolerance and induction of diabetes by virus infection in viral antigen transgenic mice was reported by Ohashi and coworkers (1991). Von Herrath and coworkers (1994) investigated how virus induces a rapid or slow onset insulin-dependent diabetes mellitus in two distinct transgenic mouse models. Von Herrath and coworkers (1995) evaluated the role of the costimulatory molecule B7-1 in overcoming peripheral ignorance in transgenic mice which expressed the glycoprotein or nucleoprotein of lymphocytic choriomeningitis virus as the self-antigen in pancreatic β -cells. Von Herrath and Holz (1997) reported that pathological changes in the islet milieu precede infiltration of islets and destruction in β -cells by autoreactive lymphocytes in a transgenic model of virus-induced IDDM. RIP-LCMV transgenic mice that express the viral glycoprotein or nucleoprotein from lymphocytic choriomeningitis virus (LCMV) under control of the rat insulin promoter (RIP) in pancreatic β -cells develop autoimmune diabetes after infection with LCMV. Upregulation of MHC class II molecules associated with the attraction/activation of antigen presenting cells to the islets occurs as soon as 2 days after LCMV inoculation of transgenic mice, clearly before CD4⁺ and CD8⁺ lymphocytes are found entering the cells. Possibilities of treatment of virus-induced autoimmune diabetes were discussed (von Herrath et al. 1997). Moritani and coworkers (1996) reported the prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes as a gene therapy model for autoimmune diabetes. Birk and coworkers (1996) generated transgenic NOD mice carrying a murine Hsp60 transgene driven by the H²E _{α} class II promoter in order to examine the hypothesis of a pathogenic role for

self-reactive cells against the stress protein Hsp60 in autoimmune destruction of pancreatic cells in the diabetes of NOD mice.

Future Developments

The employment of even more sophisticated strategies in conditional gene targeting may overcome these limitations in the future. Conditional inactivation of genes is mainly achieved by the Cre-loxP system (Lewandoski 2001). Moller (1994) and Plum and coworkers (2005) recently summarized our current knowledge about the use of knockout and transgenic animal models in diabetes research. Animal models of insulin resistance has recently been reviewed by Nandi and coworkers (2004).

K.2.6

Metabolic Systems Biology

With the completion of the many genomes, genetics is positioned to meet physiology. Argmann and coworkers (2005) predicted the coming of “systems metabolism” in mammals through the use of the mouse, as a model system to study metabolism. Building on mouse genetics with increasingly sophisticated clinical and molecular phenotyping strategies has enabled scientists to now tackle complex biomedical questions, such as those related to the pathogenesis of the common metabolic disorders. The ultimate goal of such strategies will be to mimic human metabolism with the click of a mouse.

Techniques in mouse genetics and phenomics have rapidly evolved over the past decade, which with the renaissance of integrative physiology positioned scientists well for the emergence of the discipline, systems biology. Such systems biology approaches hold great promise in the area of metabolism. The efficient conversion of metabolic parts to metabolic systems will depend on the integration of discovery-based and hypothesis-driven phenogenomic approaches, which rely on the study of mouse models derived from both reverse- and forward genetic strategies. A stable marriage of *in silico* with wet biology that allows for reiterative testing of the computationally identified networks will be crucial for the success of systems biology approaches. Such integrated approaches will become indispensable future resources for drug target identification as well as drug discovery and evaluation. It is expected that such insight into complex metabolic networks will ultimately translate into better therapeutic strategies for metabolic diseases.

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K.3 Measurement of Blood Glucose-Lowering and Antidiabetic Activity

K.3.1 Hypoglycemic Effects

K.3.1.1 Blood Glucose-Lowering Effect in Rabbits

PURPOSE AND RATIONALE

The rabbit has been used since many years for standardization of insulin (see K.4.1). Therefore, it has been chosen as primary screening model for screening of blood glucose lowering compounds as well as for establishing time-response curves and relative activities (Bänder et al. 1969, Geisen 1988).

PROCEDURE

Groups of 4–5 mixed breed rabbits (e. g. Hoe:BASK, SPFWiga) of either sex weighing 3.0–4.5 kg are used. For insulin evaluation, food is withheld overnight. For evaluation of sulfonylureas and other blood glucose lowering agents the animals are on a normal diet (e. g. Era mixed feed 8300) prior to the experiment. The animals are gently placed into special restraining boxes allowing free access to the rabbit's ears.

Oral blood glucose lowering substances are applied by gavage in 1 ml/kg of 0.4% starch suspension or intravenously in solution. Several doses are given to different groups. One control group receives the vehicle only. By puncture of the ear veins, blood is withdrawn immediately before and 1, 2, 3, 4, 5, 24, 48, and 72 h after treatment. For time-response curves values are also measured after 8, 12, 16, and 20 h. Blood glucose is determined in 10 μ l blood samples with the hexokinase enzyme method (Glucoquant test kit).

Evaluation

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects at a time interval are calculated using the trapezoidal rule. The values of the experimental group are compared statistically with the *t*-test or the WILCOXON test for each time interval with those of the control group. Differences between several treated groups and the control group are tested using a simultaneous comparison according to Dunnett or Nemenyi/Dunnett. Dose dependencies and relative activities are determined by means of linear regression analysis after Fieller (1944) and Sidak (1967). All data

for statistical comparisons have to be tested for homogeneity of variances according to Levene (1960) and for normal distribution according to Shapiro and Wilk (1965). In the case of regression analyses, the lines are additionally tested for parallelism according to Tuckey (1966) and for linearity according to Scheffé (1959). The level of significance for all procedures is chosen as 5%.

MODIFICATIONS OF THE METHOD

For special purposes the effect of blood sugar lowering agents is studied in glucose loaded animals. Rabbits of either sex weighing 3.0–4.5 kg are treated either once (0.5 h after test compound) or twice (0.5 and 2.5 h after test compound) orally with 2 g glucose/kg body weight in 50% solution.

A biological assay of insulin preparations in comparison with a stable standard using the blood sugar lowering effect in rabbits has been proposed by Harrison and coworkers (1925).

The biological assay of insulin using the blood sugar lowering effect in rabbits has been until recently the official assay in several pharmacopoeias, such as European Pharmacopoeia, 2nd edn 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988; United States Pharmacopoeia 23 and The National Formulary 18, 1995.

The rabbit blood glucose bioassay as well as the mouse convulsion assay and the mouse glucose assay were used for establishing international standards for highly purified human, porcine and bovine insulins (Bristow et al. 1988).

In several pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd edn 1997; but the rabbit blood sugar method is still valid in the United States Pharmacopoeia USP 24, 2000).

PROCEDURE

Four groups of at least 6 randomly distributed rabbits weighing at least 1.8 kg are kept in the laboratory and maintained on a uniform diet for not less than one week before use in the assay. About 24 h before the test each rabbit is provided with an amount of food that will be consumed within 6 h. The same feeding schedule is followed before each test day. During the test all food and water is withheld until the final blood sample has been taken. The rabbits are placed into comfortable restraining cages to avoid undue excitement.

Immediately before use two solutions of the standard preparation are made, containing 1 unit and 2 units of insulin per ml, respectively, and two di-

lutions of the preparation being examined which, if the assumption of potency is correct, contain amounts of insulin equivalent to those in the dilutions of the standard preparation. As diluent, a solution is used of 0.1–0.25% w/v of either m-cresol or phenol and 1.4 to 1.8 w/v of glycerol being acidified with hydrochloric acid to a pH between 2.5 and 3.5.

Each of the prepared solutions is injected subcutaneously to one group of rabbits, using the same volume, which should usually be between 0.3 and 0.5 ml for each rabbit, the injections being carried out according to a randomized block design. Preferably on the following day, but in any case not more than 1 week later, each solution is administered to a second group of rabbits following a twin crossover design. One hour and 2.5 h after each injection a suitable blood sample is taken from the ear vein of each rabbit. Blood sugar is determined by a suitable method, preferably using glucose oxidase.

EVALUATION

The results of the assay are calculated by standard analytical methods (e. g., USP 23, 1995).

CRITICAL ASSESSMENT OF THE METHOD

The classical bioassay based on blood-sugar lowering activity in rabbits has been replaced by chemical methods in some pharmacopoeias (Underhill et al. 1994), but is still included in USP 24 (2000) and will be still necessary for evaluation of synthetic insulin derivatives.

MODIFICATIONS OF THE METHOD II

An assay of insulin activity after intraperitoneal injection in rats has been described by Rafaelsen and coworkers (1965) and Young (1967). Shults and coworkers (1994) reported as one of the first on an implantable potentiostat-radiotelemetry system for *in vivo* sensing of glucose, implanted into the paravertebral thoracic subcutaneous tissue of a dog. An enzyme electrode sensor measures the oxidation current of hydrogen peroxide formed by the stoichiometric conversion of the substrate glucose and oxygen as a cofactor in an immobilized glucose oxidase layer. Salehi and coworkers (1996) described the development of a compact, low power, implantable system for *in vivo* monitoring of oxygen and glucose concentrations.

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K.3.1.2

Blood Glucose-Lowering Effect in Rats

PURPOSE AND RATIONALE

Rats are used for screening as well as for quantitative evaluation of blood glucose lowering agents.

PROCEDURE

Male Wistar rats (e. g. Hoe:WISKf, SPF 71) weighing 180–240 g are kept on standard diet (e. g. Altromin 1324). Groups of 4–7 non-fasted animals are treated orally or intraperitoneally with various doses of the test compounds suspended in 0.4% starch suspension. One control group receives the vehicle only. Blood is withdrawn from the tip of the tail immediately before, and 1, 2, 3, 5, and 24 h after administration of the test compound. Blood glucose is determined in 10 µl blood samples with the hexokinase enzyme method (Glucoquant test kit).

EVALUATION

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects over a time period are calculated using the trapezoidal rule. Statistical evaluation is performed as described for tests in rabbits.

MODIFICATIONS OF THE METHOD

Studies in Glucose-Loaded Rats

For special purposes the effect of blood sugar lowering agents is studied in glucose loaded animals. One g glucose/kg body weight is given in a 50% solution either orally 5 min after oral administration or subcutaneously 5 min after intraperitoneal administration of the test compound.

Studies in Streptozotocin-Diabetic Rats

Male Wistar rats (e. g. Hoe:WISKf, SPF 71 strain) weighing 170–220 g are kept on standard diet (e. g. Altromin 1324). Ten–fourteen days prior to the study they are injected with 60 mg/kg streptozotocin (Calbiochem) intravenously. Blood sugar levels rise from 5.5–6.0 mmol/l up to 25.0–28.0 mmol/l and glucosuria occurs. Plasma insulin levels fall below 4 µU/ml. Compounds which release insulin from pancreatic islets as sole hypoglycemic activity are not effective in rats with severe streptozotocin induced diabetes.

K.3.1.3**Blood Glucose-Lowering Effect in Mice****PURPOSE AND RATIONALE**

Eneroth and Ahlund (1968, 1970a and b) recommended a twin crossover method for bioassay of insulin using blood glucose levels in mice instead of hypoglycemic seizures giving more precise results. This test was induced into the British Pharmacopoeia 1980 and continued up to 1988. Moreover, the test is included as alternative in the European Pharmacopoeia, 2nd edn 1980; and in Deutsches Arzneibuch, 9. Ausgabe, 1986.

PROCEDURE

Non-fasting mice of the same strain and sex are used having body masses such that the difference between the heaviest and lightest mouse is not more than 2 g. The mice are assigned at random to four equal groups of not less than 10 animals. Two dilutions of a solution of the substance or of the preparation to be examined and 2 dilutions of the reference solution are prepared using as diluent 0.9% NaCl solution adjusted to pH 2.5 with 0.1 N hydrochloric acid and containing a suitable protein carrier. In a preliminary experiment, concentrations of 0.02 IU and 0.10 IU are tested. Each of the prepared solutions (0.1 ml/10 g body weight) is injected subcutaneously to one group of mice according to a randomized block design. Not less than 2.5 h later, each solution is administered to a second group of mice following a twin crossover design. Exactly 30 min after each injection, a sample of 50 µl of blood is taken from the orbital venous sinus of each mouse. Blood glucose concentration is determined by a suitable method, such as described by Hoffman (1937).

EVALUATION

The potency is calculated by the usual statistical methods for the twin-cross-over assay.

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K.3.1.4**Blood Glucose-Lowering Effect in Dogs****PURPOSE AND RATIONALE**

Besides experiments in rats and rabbits studies in dogs are necessary to predict the effect of a new compound in man due to differences in species related metabolism.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are kept on standard diet (e. g. Erka mixed feed 8500). Food is withdrawn 18 h prior to the administration of the test compound which is given either orally or intravenously in various doses. Control animals receive the vehicle only. Blood is collected at different time intervals up to 48 h. Blood glucose is determined with the hexokinase enzyme method (Glucoquant test kit) and plasma insulin with an immunological method (Riagnost-kit).

EVALUATION

Average blood sugar values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects over a time period are calculated using the trapezoidal rule. Similarly, plasma insulin levels are plotted versus time and compared with control values. Statistical evaluation is performed as described for tests in rabbits.

MODIFICATIONS OF THE METHOD**Studies in Pancreatectomized Dogs**

The surgical technique of pancreatectomy in dogs is described in Sect. K.1.0.1. The animals are pancreatectomized up to 2–3 years prior to the study. They are kept on dry feed (Vipromix) together with 2–3 g pancreatic enzymes (Vivaler). Insulin is substituted with a single daily subcutaneous dose of 32 IU Insulin Ultratard HM. For substitution of vitamin D an intramuscular dose of 1 ml Vigantol is given every 3 months.

On the day before the study, the animals receive 32 IU of the shorter acting Basal-H insulin. This insulin is administered at the same time when food and test compound are given in the morning. The test drug

is applied as oral suspension in tap water. Blood glucose is determined before and up to 6 h after treatment in hourly intervals. Control animals receive tap water only.

Studies in Alloxan-Diabetic Dogs

Chemical diabetes can be induced by a single intravenous dose of 60 mg/kg alloxan. Afterwards, the animals receive infusions of 1000 ml 5% glucose together with 10 IU Regular Insulin via a jugular vein catheter daily during one week and canned food ad libitum. Thereafter, a single dose of 28 IU Insulin Ultratard HM is given daily and the animals are fed commercial diet (Altromin pellets). On the day before the study, the dogs receive 28 IU of the shorter acting Basal-H insulin. This insulin is given at the same time as food and test compound in the morning. The test drug is applied as oral suspension in tap water. Blood glucose is determined before and up to 6 h after treatment in hourly intervals. Control animals receive tap water only.

Continuous Blood Glucose Monitoring

A device for continuous blood glucose monitoring and infusion in freely mobile dogs was described by Geisen and coworkers (1981, 1988) and Bänder and coworkers (1969).

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K.3.1.5

Blood Glucose-Lowering Effect in Other Species

Instead of rats male guinea pigs (e. g., Pirbright white, Hoe:DHPK, SPF Lac) weighing 250–380 g can be used. Blood is withdrawn by puncture of ear veins before, and 1, 3, and 5 h after administration of the test compound or the vehicle. Blood sugar determinations and statistical evaluations are performed as described for rabbits and rats.

Genetically obese and diabetic yellow KK mice have been used by Sohda and coworkers (1990) for evaluation of hypoglycemic activity of potential an-

tidabetic drugs. Gill and Yen (1991) studied the effect of ciglitazone in obese-diabetic viable yellow mice (VY/Wfl-A^{vy/a}).

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K.3.2

Euglycemic Clamp Technique

PURPOSE AND RATIONALE

The euglycemic glucose clamp technique has provided a useful method of quantifying *in vivo* insulin sensitivity in humans (DeFronzo et al. 1979). In this technique a variable glucose infusion is delivered to maintain euglycemia during insulin infusion. Whole-body tissue sensitivity to insulin, as determined by net glucose uptake, can be quantitated under conditions of near steady state glucose and insulin levels. Kraegen and coworkers (1983, 1985) developed the euglycemic glucose clamp technique for use in the intact conscious rat.

PROCEDURE

Male Wistar rats weighing 150–200 g are fasted overnight and anesthetized with pentobarbital (40 mg/kg, i.p.). Catheters are inserted into a jugular vein and a femoral vein for blood collections and insulin and glucose infusion, respectively. To evaluate the insulin action under physiological hyperinsulinemia (steady state plasma insulin concentration during the clamp test around 100 μ U/dl), and maximal hyperinsulinemia (under which maximal insulin action may appear) two insulin infusion rates, 6 and 30 mU/kg/min, are used. The blood glucose concentrations are determined from samples collected at 5-min intervals during the 90-min clamp test. The glucose infusion rate is adjusted so as to maintain the blood glucose at its basal level during the clamp test. The final glucose infusion rate is calculated from the amount of glucose infused for the last 30 min (from 60 to 90 min after start of the clamp) in which the blood glucose levels are in a steady state. The glucose metabolic clearance rate is obtained by dividing the glucose infusion rate by the steady state blood glucose concentration. The steady state plasma insulin concentration is calculated from the insulin concentrations at 60 and 90 min after the start of the

clamp. At the start and end of the euglycemic clamp test, free fatty acid concentration is also determined and the free fatty acid suppression rate is calculated.

EVALUATION

All values are analyzed by one-way ANOVA. When the steady state plasma insulin is maintained at sub-maximal concentration by the euglycemic clamp technique, the glucose infusion rate and glucose metabolic clearance rate value are considered to reflect the state of receptor binding levels in the peripheral tissue as an index for insulin sensitivity. Under maximal hyperinsulinemia these values are thought to reflect the state of the enzymes and glucose transport system activated after the binding to receptors, indicating mainly insulin responsiveness.

MODIFICATIONS OF THE METHOD

Burnol and coworkers (1983) used the euglycemic insulin clamp technique coupled with isotopic measurement of glucose turnover to quantify insulin sensitivity in the anesthetized rat.

Bryer-Ash and coworkers (1995) used this technique to demonstrate reduction of insulin sensitivity by amylin corresponding to reduced insulin receptor kinase activity.

The effects of counterregulatory hormones on insulin-induced glucose utilization by individual tissues in rats, using the euglycemic hyperinsulinemic clamp technique combined with an injection of 2-[1-³H]-deoxyglucose, were studied by Marfaing and coworkers (1991).

Lang (1992) determined the insulin-mediated glucose uptake in normal and streptozotocin-diabetic rats using the euglycemic-hyperinsulinemic clamp technique.

Hirshman and Horton (1990) reported increased insulin sensitivity and responsiveness in peripheral tissues of the rat after glyburide as determined by the glucose clamp technique.

Lee and coworkers (1994) studied the metabolic effects of troglitazone on fructose-induced insulin resistance with the euglycemic hyperinsulinemic clamp technique in rats.

Tominaga and coworkers (1992, 1993) studied the influence of insulin antibodies in anesthetized rats and of thiazolidinediones on hepatic insulin resistance in streptozotocin-induced diabetic rats by the glucose clamp technique.

Gelardi and coworkers (1991) used the hyperinsulinemic-euglycemic clamp technique to evaluate

the insulin sensitivity in the obese offspring of streptozotocin-induced mildly hyperglycemic rats.

Hulman and coworkers (1993) studied insulin resistance in the conscious spontaneously hypertensive rat with the euglycemic hyperinsulinemic clamp technique.

Cheung and Bryer-Ash (1994) described a modified method for the performance of glucose insulin clamp studies in conscious rats under local anesthesia.

Burvin and coworkers (1994) developed a modification of the euglycemic insulin clamp technique and used it to repeatedly assess *in vivo* insulin effects in awake streptozotocin-induced diabetic rats.

Xie and coworkers (1996) described a modified euglycemic clamp technique in cats.

Finegood and coworkers (1987) estimated endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps using unlabeled and labeled glucose infusates in dogs.

Xie and coworkers (1996) described an insulin sensitivity test using a modified euglycemic clamp in cats and rats. This test uses the amount of glucose required to be infused to maintain euglycemia over a 30-min period in rats and 60 min in cats following a bolus administration of insulin as the index of insulin sensitivity. Glucose levels are determined at short intervals and variable glucose infusion is used to hold glucose levels within a few percentage points of the basal pre-test glucose level. A new blood sampling technique is described that allows each insulin sensitivity test to be carried out using a total of only 0.5 ml of blood.

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K.3.3

Hypoglycemic Seizures in Mice

PURPOSE AND RATIONALE

The biological assay of insulin using hypoglycemic seizures in mice has been suggested already in 1923 by Fraser. The biological standardization of insulin using the mouse convulsion method has been published in detail by the Health Organisation of the League of Nations in 1926 (Trevan and Boock, Hemmingsen and Krogh) and has been until recently the official assay in several pharmacopoeias, such as European Pharmacopoeia, 2nd edn 1980; Deutsches Arzneibuch 1986; British Pharmacopoeia 1988. In most pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia, 3rd edn 1997).

PROCEDURE

Ninety-six mice of either sex (but not of mixed sexes) weighing 20 ± 5 g are randomly distributed into

4 groups. The mice are deprived of food 2–20 h immediately preceding the test. Solutions of the insulin standard and of the test preparation containing 30 and 60 milliUnits/ml are prepared by diluting the original solution with 0.9% NaCl solution, pH 2.5. 0.5 ml/20 g mouse of these solutions are injected subcutaneously. The mice are kept at a uniform temperature, between 29 and 35°C, in transparent containers within an air incubator with a transparent front. The mice are observed for 1.5 h and the number of mice is recorded that are dead, convulse or lie still for more than 2 or 3 s when placed on their backs.

EVALUATION

The percentage of mice of each group showing the afore mentioned symptoms is calculated and the relative potency of the test solution calculated using a 2 + 2 point assay.

CRITICAL ASSESSMENT OF THE METHOD

Attempts to replace the tests in mice and rabbits by *in vitro* tests, such as the rat diaphragm test, the rat epididymal fat pad test, or even the radioimmunoassay failed due to several reasons (Trethewey 1989). Nevertheless, for industrial production and for stability studies, the classical bioassays based on hypoglycemic seizures in mice or hypoglycemia in rabbits have been replaced by chemical methods (Stewart 1974, Underhill et al. 1994).

MODIFICATIONS OF THE METHOD

A modification of the mice seizure method using rotating hollow cylinders has been proposed by Young and Lewis (1947). A similar technique which increases the sensitivity of the mice seizure method has been used by Vogel (1964). The equipment consisted of seven perforated metal drums with a diameter of 15 cm. The drums were rotated in oblique position at 10 rotations per minute. Female mice weighing 16 to 20 g were deprived of feed at the afternoon before the test. On the test day, groups of 6 mice received doses of 0.5; 1.0 or 1.5 IU/kg of test preparation or standard subcutaneously and were placed after 20 min into the rotating drums. Controls received saline only. Mice with insulin-induced seizures dropped out from the rotating drum. The number of animals dropping out was counted after 15; 30; 45 and 60 min. Mice dropping out due to hypoglycemic seizures received 0.5 ml of 10% glucose solution intraperitoneally. ED_{50} values and activity ratios were calculated according to Litchfield and Wilcoxon (1949).

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K.3.4**Effects of Insulin Sensitizer Drugs****PURPOSE AND RATIONALE**

Insulin sensitizer drugs are reported to improve symptoms in patients with established type 2 diabetes (NIDDM = non-insulin dependent diabetes mellitus) (Colca 1995; Kuehnle 1996). In contrast to sulfonylureas, these compounds do not lower blood glucose in normal animals. Various animal models resembling type 2 diabetes are used for evaluation. Details of these models are described in the respective chapters. *In vitro* techniques showed an increased glucose uptake into muscle tissue and into adipocytes.

PROCEDURE***In Vivo Studies***

Chang et al. (1983) studied ciglitazone in *ob/ob* and *db/db* mice, diabetic Chinese hamsters, and normal and streptozotocin-diabetic rats.

Fujita et al. (1983) investigated the effects of ciglitazone in obese-diabetic yellow *KK* (*KK-A^y*) mice and obese Zucker-fatty rats.

Diani et al. (1984) treated C57BL/6J-*ob/ob* and C57BL/KsJ-*db/db* mice for several weeks with cigli-

tazone and studied the morphological effects on pancreatic islets.

Fujiwara et al. (1988) performed studies in *KK* and *ob/ob* mice and Zucker fatty rats.

Moreover, Fujiwara et al. (1991) studied the effects of CS-045 on glycemic control and pancreatic islet structure at a late stage of the diabetes syndrome in C57BL/KsJ-*db/db* mice.

Ikeda et al. (1990), Sohda et al. (1990) used insulin resistant animals (yellow *KK* mice, Zucker fatty rats, and obese Beagle dogs with moderate insulin resistance).

Gill and Yen (1991) studied the effects on endogenous plasma islet amyloid polypeptide and insulin sensitivity in obese-diabetic viable yellow mice.

Hofmann et al. (1991, 1992) treated insulin resistant *KK^{Ay}* mice.

Stevenson et al. (1991) studied the effects of englitazone in nondiabetic rats and found no overt hypoglycemia but an enhancement of insulin action.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activating insulin receptor kinase in genetically obese Wistar fatty rats treated with various doses of pioglitazone.

Sugiyma et al. (1992) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

Tominaga et al. (1993) used the glucose clamp-technique in streptozotocin-induced diabetic rats.

Yoshioka et al. (1993) found antihypertensive effects in obese Zucker rats.

Lee et al. (1994) studied the metabolic effects on fructose-induced insulin resistance in rats.

Apweiler et al. (1995) administered BM 13.09143 to lean and obese Zucker rats and performed hyperinsulinemic-euglycemic clamp studies in these animals.

Fujiwara et al. (1995) found a suppression of hepatic gluconeogenesis in long-term troglitazone treated diabetic *KK* and C57BL/ksJ-*db/db* mice.

Lee and Olefsky (1995) studied the effects of troglitazone in normal rats with the euglycemic glucose clamp technique.

In Vitro Studies

Kirsch et al. (1984) found a reversal of cAMP-induced post-insulin receptor resistance in rat adipocytes *in vitro*.

Ciaraldi et al. (1990) performed *in vitro* studies using cultured hepatoma cells (Hep G2) and muscle cells (BC3H-1) and found an increased glycogen synthase I activity in both cell types.

Murano et al. (1994) found a stimulation of fructose-2,6-bisphosphate production in rat hepatocytes.

Kellerer et al. (1994) reported the prevention of glucose-induced insulin resistance of insulin receptor in rat-1 fibroblasts.

Bader et al. (1993) found an increased [³²P]incorporation in the 95 kD β -subunit of the insulin receptor and an increased phosphorylation of the synthetic substrate Poly[GluNa4:1Tyr] in receptors isolated from skeletal muscle of obese Zucker rats treated with CS 045.

Teboul et al. (1995) found that thiazolidinediones convert myogenic cells (C₂C₁₂N myoblasts, a subclone of the C₂C₁₂ cell line) into adipose-like cells. Thiazolidinediones or fatty acids prevented the expression of myogenin, α -actin, and creatine kinase and abolished the formation of multinucleated myotubes. In parallel, these treatments induced the expression of a typical adipose differentiation program including acquisition of adipocyte morphology and activation of adipose-related genes.

Tafuri (1996) reported that troglitazone enhanced the rate and percent differentiation of fibroblasts to adipocytes. Basal glucose transport and synthesis of GLUT1 transporter messenger RNA were increased.

Stevenson et al. (1990) examined the effects of racemic englitazone (CP 68722) in adipocytes and soleus muscles from *ob/ob* mice and in 3T3-L1 adipocytes. Administration of the drug in various doses lowered plasma glucose and insulin dose-dependently without producing frank hypoglycemia in either diabetic or nondiabetic animals. Basal and insulin-stimulated lipogenesis were enhanced in adipocytes from *ob/ob* mice. Glycogenesis and basal glucose oxidation in isolated soleus muscles were stimulated.

Kreutter et al. (1990) used 3T3-L1 adipocytes and found a stimulation of 2-deoxyglucose uptake.

Masuda et al. (1995) found an insulinotropic mechanism distinct from glibenclamide in isolated rat pancreatic islets and HIT cells.

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- tor-activated receptors α and γ (Kliwer et al. 1997). PPAR γ is a central regulator of adipocyte gene expression and differentiation (Tortonoz et al. 1995; Brun et al. 1996; Wu 1998, Lowell 1999). Thiazolidinedione derivatives which are antidiabetic agents are potent and selective activators of PPAR γ (Young et al. 1997; Henke et al. 1998, Murakami et al. 1998; Reginato et al. 1998; Ribon et al. 1998, Vázquez et al. 2002).. Berger et al. (1996) found a correlation of antidiabetic actions of thiazolidinediones in *db/db* mice with the conformational change in peroxisomal proliferator-activated receptor- γ : Murphy and Holder (2000) suggested a therapeutic potential of PPAR γ agonists in the treatment of inflammatory diseases and certain cancers.
- Steppan et al. (2001), Berger (2001) showed that adipocytes secrete a signaling molecule which they called resistin (for resistance to insulin). Circulating resistin levels in mice are decreased by thiazolidinediones and are increased in diet-induced and genetic forms of obesity. On the other hand adipocytes secrete another adipocytokine called adiponectin. Serum levels of adiponectin correlate with insulin sensitivity and are increased by the treatment with thiazolidinediones (Choi et al. 2005, Do et al. 2006).

PROCEDURE

Plasmids

The pSG5-haPPAR γ 1 expression construct is generated by inserting the hamster PPAR γ complementary DNA into the EcoRI site of the pSG5 expression vector (Stratagene, La Jolla, CA). As reporter construct, pPPRE-chloramphenicol acetyltransferase (CAT) is used containing two copies of the peroxisomal proliferator response element from the enhancer region of the murine acyl coenzyme A-oxidase gene adjacent to the glutathione-S-transferase minimal promoter and the CAT gene.

Cell Culture and Transfections

COS-1 cells are seeded at 2.1×10^5 cells per dish in 35-mm dishes (for transactivation assays) and 3×10^6 cells/dish in 150-mm dishes (for binding assays) in DMEM (high glucose) containing 10% charcoal-stripped fetal calf serum, nonessential amino acids, 100 U/ml penicillin G, and 100 μ g/ml streptomycin sulfate at 37°C in a humidified atmosphere of 10% CO₂. After 24 h, transfections are performed with Lipofectamine (Life Technologies, Gaithersburg, MD). For transactivation experiments, transfection mixes are used containing 1 μ g receptor expression vector, and 1 μ g pCH110 (Pharmacia, Piscataway, NJ)

K.3.5

Effects of Thiazolidinediones on Peroxisome Proliferator-Activated Receptor- γ

PURPOSE AND RATIONALE

Peroxisome proliferator-activated receptors (PPARs) compose a subfamily of the nuclear hormone receptors. Three distinct PPARs, termed α , δ , and γ , each encoded by a separate gene and showing a distinct tissue distribution pattern, have been described (Keller and Wahli 1993; Green 1995; Devchand et al. 1996; Lemberger et al. 1996; Schoonjans et al. 1996a, b, 1997, Willson et al. 2000), Walczak and Tontomoz 2002). Ligands that induce the transcriptional activity of PPAR α and γ have been identified (Forman et al. 1995; Devchand et al. 1996; Lehmann 1995). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome prolifera-

as an internal control and, for binding studies 20 µg receptor expression vector.

Binding Assay

Transfected cells are grown for 48 h after transfection with the receptor expression vector. Receptor preparation is performed according to Tilley et al. (1989). Cell lysates containing receptor are prepared in TEGM (10 mM Tris-HCl, pH 7.2; 1 mM EDTA; 10% glycerol; 7 µl/100 ml β-mercaptoethanol; 10 mM Na molybdate; 1 mM dithiothreitol; 5 µg/ml aprotinin; 2 µg/ml leupeptin; 2 mg/ml benzamide; and 0.5 mM phenylmethylsulfonylfluoride). Plates are placed on ice, rinsed with TEG (10 mM Tris-HCl, pH 7.2; 50 mM EDTA; and 10% glycerol), and scraped into 0.5 ml TEGM. The material is pooled, frozen in liquid nitrogen to lyse the cells, and thawed on ice. The lysate is centrifuged at 22,000g for 20 min at 4°C to remove the debris and stored frozen (-80°C) until use. For each assay, an aliquot of receptor-containing lysate (0.1–0.25 mg protein) is incubated with 10 nM ditiated AD-5075 (21 Ci/mmol) with or without test compound for ~16 h at 4°C in TEGM (300 µl final volume). Unbound ligand is removed by incubation on ice for ~10 min after the addition of 200 µl dextran/gelatin-coated charcoal. After centrifugation at 3000 rpm for 10 min at 4°C, 200-µl aliquots of supernate are counted in an liquid scintillation counter.

PPAR binding assays using gel filtration to separate the bound radioactivity ligand from the free ligand (Kliwer et al. 1997) have been replaced by the scintillation proximity assay (SPA) which increases the throughput of PPAR competition assays (Nichols et al. 1998, Elbrecht et al. 1999). In this technique the ligand binding domain is labeled with biotin and immobilized on streptavidin-modified scintillation proximity beads. When a potential radioligand of PPARγ, binds to the PPARγ ligand binding domain, light, which leads to a detectable signal, is emitted. Competition with a non-labeled ligand avoids the binding of the radioligand to the PPARγ ligand binding domain and no signal is emitted. With this binding assay, there is no need to separate free radioligands from bound ligands, thus simplifying the process.

Su et al. (1999) described the use of a PPARγ-specific monoclonal antibody to demonstrate thiazolidinediones induced PPARγ receptor expression *in vitro*.

Transactivation Assay

After transfection, cells are incubated for 48 h in culture medium with or without increasing concentrations of test compounds. Cell lysates are produced

using reporter lysis buffer (Promega Corp., Madison, WI). CAT activity is determined using radiolabeled butyryl CoA as substrate in a diffusion based assay. β-Galactosidase activity is determined according to Holtons and Yoshimura (1989).

Lipogenesis Assay

PPARγ plays an important role in the regulation of cell differentiation (Lowell 1999). Adipogenesis requires combined high levels of PPARγ and other transcription factors such as CAAT/enhancer binding protein (C/EBP) and adipocyte determination and differentiation-dependent factor 1/sterol regulatory binding protein (ADD-1/SREBP-1). PPARγ plays an essential role in the molecular control of adipogenesis. Ectopic expression of PPARγ induces adipocyte differentiation (Tontonoz et al. 1994), whereas in the absence of PPARγ adipocytes fail to develop (Lowell 1999). Adipocyte differentiation leads to expression of adipocyte-specific genes, such as aP2 (Tontonoz et al. 1994) or lipoproteinlipase (Schoonjans et al. 1996c) which are under the control of PPARγ. The lipogenesis assay, based on preadipocytes differentiation to adipocytes, where PPARγ plays an important role, has been used to select PPARγ agonists for *in vivo* studies. Henke et al. (1998) tested compounds for their ability to promote differentiation of C3H10T1/2 stem cells via measurement of glucose incorporation into total lipid of the cells.

Protease Digestion Assay

The protease digestion assay is performed according to the method of Allan et al (1992) with minor modifications. The plasmid pSG5-haPPARγ1 is used to synthesize ³⁵S-radiolabeled PPARγ1 in a coupled transcription/translation system. The transcription/translation reactions are subsequently aliquoted into 22.5-µl volumes, and 2.5 µl PBS with or without a thiazolidinedione are added. The mixtures are incubated for 20 min at 25°C, separated into 4.5-µl aliquots, and 0.5 µl distilled water or distilled water-solubilized trypsin are added. The protein digestions are allowed to proceed for 10 min at 25°C, then terminated by the addition of 20 µl denaturing gel loading buffer and boiling for 5 min. The products of the digestion are separated by electrophoresis through a 1.5-mm 12% polyacrylamide-SDS gel. After electrophoresis, the gels are fixed in 10% acetic acid-40% methanol for 30 min, treated in EN³HANCE for an additional 30 min. and dried under vacuum for 2 h at 80°C. Autoradiography is then performed to visualize the radiolabeled digestion products.

EVALUATION**Binding Assays**

For binding assays, competition curves are generated by incubation of 10 nM [³H]AD-5075 with hamster PPAR γ 1 produced by transient transfection of COS-1. The percentage of ligand bound after incubation in the presence of the indicated concentration of each unlabeled compound for 16 h is plotted.

Activation Assays

For activation assays of PPAR γ in COS-1 cells transiently cotransfected with pSG5-haPPAR γ 1 and pPPRE-CAT, normalized CAT activity is plotted after incubation in the presence of the indicated concentration of each compound for 48 h.

Lipogenesis Assays

The lipogenic activity of PPAR agonists can be assessed by determining radioactive glucose uptake into the total lipids of cells. The amount of glucose incorporation into lipids in these cells provides a measure of cell differentiation. Moreover, the expression of adipocyte marker genes (aP2, lipoprotein lipase, leptin) and the accumulation of triglycerides in the cytoplasm confirmed by oil red O staining are other methods to evaluate the lipogenic activity of PPAR γ agonists.

Protease Digestion Assay

For evaluation of the protease digestion assay, the partially protease-resistant conformation product of PPAR γ is visualized by autoradiography on SDS-PAGE after incubation with the thiazolidinedione and increasing concentrations of trypsin.

MODIFICATIONS OF THE METHOD

Berger et al. (1999) reported distinct biological effects of PPAR γ and PPAR δ ligands.

Desvergne and Wahli (1999) reviewed nuclear control of metabolism by peroxisome proliferator-activated receptors.

Lin et al. (1999) reported a fluorescence-based method for investigating the interactions of PPAR with ligands.

Brown et al. (2001) described identification of a subtype selective human PPAR α agonist through parallel-array synthesis.

Xu et al. (2001) investigated the structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors.

Lee et al. (2003) reviewed metabolic disorders and peroxisome proliferator-activated receptors.

Ram (2003) underlined the therapeutic significance of peroxisome proliferator-activated receptor modulators in diabetes.

Norris et al. (2003) found that muscle-specific PPAR γ -deficient mice develop increased adiposity and insulin resistance but respond to thiazolidines.

Vikramadithyan et al. (2003) studied a peroxisome proliferator-activated receptor alpha (PPAR α) and PPAR γ agonist as body weight lowering, hypolipidemic and euglycemic agent. HEK 293 cells were used to conduct the reporter-based transactivation of PPAR α and PPAR γ .

Matsusue et al. (2003) found that liver-specific disruption of PPAR γ in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes.

Stanley et al. (2003) studied subtype-specific effects of peroxisome proliferator-activated receptor ligands on co-repressor activity.

Wurch et al. (2002) described pharmacological analysis of wild-type α , γ and δ subtypes of the human peroxisome proliferator-activated receptor.

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K.3.6

Antidiabetic Effects of Liver X Receptor Agonists

PURPOSE AND RATIONALE

The nuclear receptors liver X receptor (LXR) α and LXR β which are sensors of cholesterol metabolism and lipid biosynthesis are also regulators of inflammatory cytokines, suppressors of hepatic glucose production, and involved in different cell-signaling pathways. LXR α is a target gene of the peroxisome proliferator-activated receptor- γ , a target of drugs used in treating elevated levels of glucose seen in diabetes (Steffensen and Gustafsson 2004). Furthermore, insulin induces LXR α in hepatocytes, resulting in increased expression of lipogenic enzymes and suppression of key enzymes in gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK). Mukherjee et al. (1997) observed sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Treatment of diabetic rodents with a LXR α agonist resulted in dramatic reduction of plasma glucose (Cao et al. 2003).

PROCEDURE

Oral Glucose Tolerance Study in *fa/fa* Rats

Obese insulin-resistant female Zucker (*fa/fa*) rats, 10 weeks of age, are orally gavaged for 9 days with either vehicle of the LXR α agonist. Eight h after the last dose, animals are fasted overnight and on the following morning subjected to an oral glucose tolerance test. Blood was obtained from the animals via the tail vein at time 0 and times 15, 30, 60, and 120 min after an oral glucose challenge (2.5 g of glucose/kg body weight). Plasma glucose and insulin levels are analyzed on all samples, and the results are expressed as the product of glucose AUC and insulin AUC.

Glucose Output with ZDF Rat Liver Slices

Precision-cut liver slices are generated from control, treated for 7 days with the LXR α agonist after an overnight fast. After preincubation and wash phases, the slices are incubated for 2 h at 29° C in Krebs-Henseleit bicarbonate buffer containing 40 mM mannitol in either the presence or absence of 10 mM lactate. Incubation media glucose levels are assessed at the 2-h time by subtracting the basal rate of glucose output per gram liver tissue from the substrate-stimulated rate of glucose output per gram of liver tissue.

Nuclear Run-On Experiment

A nuclear run-on experiment (Cao et al. 2002) is performed in the livers of diabetic *db/db* mice treated for 7 days with the LXR α agonist. Mouse liver nuclei are isolated (Schibler et al. 1983) and *in vivo* elongation reaction is performed (Goldman et al. 1985). The radiolabeled RNA is then subjected to slot blot (Mauvieux et al. 1998) in probes.

mRNA Measurement

Total RNAs are prepared from frozen tissue samples or cells with TRIzol reagent (Invitrogen) or Qiagen RNA prep kit. Mouse phosphoenolpyruvate carboxykinase and G6P mRNA are measured by RNase protection assay and quantified with a Molecular Dynamics Phosphorimager Model 51. Rat mRNA is subjected to reverse transcription reactions using the Omniscript reverse transcriptase kit (Qiagen). The resulting cDNA is amplified using TaqMan 2 \times PCR master mix (Applied Biosystems). The PCR products are detected in real time using an ABI-7900HT sequence detection system (Applied Biosystems). The rat PEPCK branched cDNA is analyzed according to Burris et al. (1999).

CRITICAL ASSESSMENT OF THE METHOD

Chisholm et al. (2003) reported that a LXR ligand induces severe lipogenesis in db/db diabetic mice.

MODIFICATIONS OF THE METHOD

Stulnig et al. (2002a, 2002b) concluded that LXR ligands mediate beneficial metabolic effects in insulin resistance syndromes including type 2 diabetes by interfering with peripheral glucocorticoid activation.

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K.4**Measurement of Insulin and Other Glucose-Regulating Peptide Hormones****K.4.1****Radioimmunoassays for Insulin, Glucagon and Somatostatin****PURPOSE AND RATIONALE**

Insulin activity is an important laboratory parameter in the clinical evaluation of several diseases such as diabetes mellitus types I and II, states of impaired glucose tolerance, and insulin-producing tumors (insulinomas), where the insulin secretion released from pancreas β -cells is altered.

The first description of an immunoassay of endogenous plasma insulin in man has been given by Yalow and Berson (1959, 1960). Yalow and coworkers (1960) provided evidence that the bioassays hitherto being used (isolated rat diaphragm, epididymal fat pad tissue) measure insulin-like activity but not true insulin levels in blood. Since that time, the method has been used and modified by many investigators, e. g., Grodsky and Forsham (1960), Morgan and Lazarow (1963), Hales and Randle (1963), Melani and coworkers (1965, 1967), Wright and coworkers 1968. Survey on the radioimmunoassay of insulin have been given by Ditschuneit and Faulhaber (1975), Freedlander and coworkers (1984). The introduction of radioimmunoassay (RIA) by Berson and Yalow (1959) provided a novel method for measuring insulin in plasma and serum. In order to improve reliability and practicability, various technologies have appeared in the field of immunoassays. Variety is seen in the choice of labels (radioactive, enzymatic, fluorescent, etc.), separation methods (precipitation of antigen-antibody complexes, coated solid-phase antibody technology, etc.), detection methods (spectrophotometry, fluorimetry, amperometry, potentiometry, and calorimetry), and principles such as the competitive or noncompetitive immunoassay. These immunoassay techniques have become important analytical methods in clinical chemistry laboratories for the selective detection of drugs or proteins as well as hormones such as insulin at trace levels.

PROCEDURES**Immunization**

Semisynthetic or biosynthetic human insulin is used as immunogen and as standard. Formerly, porcine insulin has been used since Yalow and Berson (1960) and subsequently many other authors have shown that

antisera raised against porcine insulin react identically with human and porcine insulin. Guinea pigs weighing 350–450 g are injected subcutaneously with 0.4 ml of an emulsion of 5 mg human insulin dissolved in 1.0 ml 0.01 N HCl and 3.0 ml complete Freund's adjuvant (Difco Laboratories). For boosting, 0.2 ml of an identically prepared emulsion is injected in monthly intervals. Fourteen days after the third booster injection, the animals are slightly anesthetized and 8–10 ml blood are withdrawn by cardiac puncture. Boosting is continued at monthly intervals and the animals are bled 2 weeks following each booster injection.

The optimal antiserum titer for use in the radioimmunoassay is determined using conditions identical to those employed in routine immunoassays. The percentage binding of $1 \mu\text{U}$ ^{125}I insulin is determined for dilutions of antisera ranging from 10^3 to 10^6 fold. The steepness of the antisera dilution curve is a measure of the affinity of the antiserum and therefore the potential sensitivity of the radioimmunoassay. Antisera with the steepest slopes, but not necessarily the highest titer, are selected for further study. The selected antisera dilutions are then run in an immunoassay using a full range of standards. A reduction in the percent ^{125}I -insulin bound to antibody from 50% (in the absence of unlabeled insulin) to 45% (in the presence of unlabeled insulin) ($B/\text{Bo} = 0.9$) is a reasonable measure of assay sensitivity.

Assay

The antibody-bound ^{125}I -insulin prepared as described above can be separated from free ^{125}I -insulin in various ways, such as by paper electrophoresis, as originally described by Yalow and Berson (1960), or by a two-antibody system, as described by Morgan and Lazarow (1963), Starr and coworkers (1979). In this method, the soluble insulin-anti-insulin complex is precipitated by an anti-guinea pig serum antibody. The following procedure is recommended (Freedlander et al. 1984):

- A buffer is prepared from a solution of 8.25 g boric acid and 2.70 g NaOH dissolved in 1 l water. After dissolving 5.0 g of purified bovine serum albumin (BSA), pH is adjusted with concentrated HCl to 8.0.
- In disposable plastic tubes, 10×75 mm, the following volumes are added:
 - 100 μl serum or standard
 - 900 μl buffer
 - 100 μl 1 mU ^{125}I -insulin in assay buffer
 - 100 μl guinea pig anti-insulin antiserum diluted in assay buffer (at a concentration to

bind 50% of the ^{125}I -insulin in the absence of unlabeled hormone)

- The mixture is incubated at 4°C for 72 h. Then, the following solutions are added:
 - 100 μl normal guinea pig serum diluted 1:400 in the assay buffer
 - 100 μl rabbit anti-guinea pig globulin serum diluted in assay buffer
- The mixture is again incubated at 4°C for 72 h and then centrifuged ($2,000 \times g$, 20 min, 4°C). The supernatant is decanted and radioactivity counted in the precipitate for 5 min.

EVALUATION

Counts in the nonspecific binding tubes are subtracted from counts in all other tubes. Data are linearized using an unweighted logit-log transformation (Rodbard and Frazier 1975). Micro-units insulin in a logarithmic scale are plotted against the ratio B/Bo ^{125}I -insulin on a logit scale. The range of B/Bo between 0.4 and 0.9 is the most suitable for determination of insulin concentration in plasma.

CRITICAL ASSESSMENT OF THE METHOD

The immunoassay of insulin as described by Yalow and Berson (1960) has been a break-through for many immunological assays of peptide hormones and other drugs. At present, not only guinea pig anti-insulin antisera, but also complete RIA kits are available from a number of commercial firms.

MODIFICATIONS OF THE METHOD

Cam and McNeill (1996) published a sensitive radioimmunoassay optimized for reproducible measurement of rat plasma insulin. Relatively small volumes (25 μl) of plasma from control, diabetic, and fasted rats can be assayed reproducibly with charcoal in the final separation step.

The hormones, insulin, glucagon and somatostatin are determined radioimmunologically: insulin with the RIAGnost-kit and rat insulin as standard; glucagon with a rabbit antibody, ^{125}I -glucagon as tracer, and polyethylene glycol as precipitant; somatostatin with a rabbit antibody, ^{125}I -tyrosyl-somatostatin as tracer and charcoal dextran for separation of free and bound hormone. At least 3 experiments per concentration are performed. The values of each collection period of 1 min are averaged and plotted vs time. The effects of the test compound (increase or decrease of the secreted hormones) are compared with the control periods and the effect of elevated glucose.

K.4.2**Bioassay for Glucagon****PURPOSE AND RATIONALE**

Glucagon is a 29-amino-acid, single-chain polypeptide which is secreted by the α -cells in the islets of Langerhans. It is synthesized from proglucagon, a 180-amino-acid precursor (Bell et al. 1983). Proglucagon is processed in islet and intestinal cell lines (Tucker et al. 1996). Glucagon interacts with a 60-kDa glycoprotein receptor on the plasma membrane of target cells (Sheetz and Tager 1988).

A biological assay of glucagon is described in the British Pharmacopoeia 1988. The potency of glucagon is estimated by comparing its hyperglycemic activity with that of the standard preparation of glucagon using the rabbit blood sugar assay as performed for insulin determinations.

PROCEDURE

Rabbits of either sex, weighing 1.8 to 2.8 kg are maintained under uniform conditions and an adequate uniform diet for at least one week. Forty-eight hours before the beginning of the test, each rabbit is injected with 1 ml of cortisone acetate injection. The animals are deprived of food, but not water, from 16 h before each test day until the withdrawal of the last blood sample on that day. The rabbits are randomly distributed into 4 groups of at least 6 animals.

The standard preparation to be used is the 1st International Standard for Glucagon, porcine, established in 1973, consisting of freeze-dried porcine glucagon with lactose and sodium chloride (supplied in ampoules containing 1.49 Units), or another suitable preparation the potency of which has been determined in relation to the International Standard.

The entire contents of one ampoule of the standard preparation are reconstituted with 2 ml of saline solution, acidified to pH 3.0 with hydrochloric acid and diluted with the same solvent to a convenient concentration, for example 100 milliUnits per ml. Two dilutions are made containing 24 and 6 milliUnits per ml, respectively, and at the same time two dilutions are made of the preparation being examined. The rabbits are injected subcutaneously with doses of 1 ml of each of the four solutions, giving the doses in random order following a twin cross-over design on two consecutive days at the same time each day. At 20 and 60 min after injection, a blood sample is taken from a marginal ear vein of each rabbit. Blood glucose concentrations are determined using a suitable method, such as the glucose-oxidase procedure.

EVALUATION

The result of the assay is calculated by standard statistical methods using the means of the two blood glucose levels found for each rabbit.

MODIFICATIONS OF THE METHOD

Glucagon can be determined by radioimmunoassay (Unger 1959, Harris et al. 1978, von Schenk 1984). Commercial kits are available.

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K.4.3**Receptor Binding and In Vitro Activity of Glucagon****PURPOSE AND RATIONALE**

The binding of glucagon to its receptor is assayed with rat liver plasma membranes (Neville 1968, Pohl et al. 1971, Goldstein and Blecher 1976). Displacement of ^{125}I -labeled glucagon is measured for synthetic glucagon analogs in comparison to natural glucagon. Cyclic AMP formation as the first step in glucagon action on liver is measured as a bioassay in liver plasma membranes.

PROCEDURE**Preparation of Membranes**

Male Sprague-Dawley rats weighing 160–200 g are decapitated and the livers rapidly removed and trimmed of fat and connective tissue. The pooled livers are placed on a pre-chilled glass plate and chopped finely with a stainless steel blade. Ten-gram portions of the mincate are suspended in 35 ml of ice-cold 1 mM NaHCO_3 and homogenized in a loose fitting Dounce homogenizer. Two homogenates are combined, brought to 500 ml with ice-cold medium, stirred magnetically for 5 min, and finally filtered once through two layers of cheesecloth. The filtrate is centrifuged ($1,500 \times g$, 10 min). Supernatant fluid is aspirated to waste using a serum pipette attached to a water pump. Homogenizations and centrifugations are continued until all of the tissue has been processed. The pooled pellets are again homogenized. The suspension is adjusted to $44 \pm 0.1\%$ (wt/wt) sucrose solution by the addition of 70% sucrose solution. Sucrose gradients are prepared in 1×3.5 -in. tubes by pipetting 26 ml of the tissue suspension followed by an overlay of 13 ml of $42.3 \pm 0.1\%$ sucrose. Centrifugation is carried out ($95,100 \times g$, 2 h). The float containing the plasma membranes can be removed by pinching the tube slightly above the float, then lifting off the float with a spoon-shaped Teflon-coated spatula. These floats are transferred to a pre-weighed plastic centrifuge tube

(50 ml), and the well-mixed suspension centrifuged at ($40,000 \times g$, 30 min). Following aspiration of the supernatant to waste, the tubes are reweighed in order to estimate the yield of plasma membranes. After addition of an equal volume of medium, the pellet is aspirated repeatedly through a 20-gauge needle fitted to a syringe. The plasma membrane suspension is distributed in 0.2 or 1.0 ml aliquots to screwcap plastic vessels for storage in liquid nitrogen.

Radioiodination of Glucagon

Three nmol glucagon are allowed to react with a 1.0 nmol sample of carrier-free Na^{125}I (2.0 mCi) in the presence of 1.5 nmol chloramine-T, added at a regular interval of 30 s (0.5 nmol each time). Reaction is terminated by addition of 0.5% sodium metabisulfite solution (Hagopian and Tager 1983). By chromatographic purification (Jørgensen and Larsen 1972), fractions of 2 ml are collected, and the monoiodinated glucagon as determined by reverse phase HPLC is stored at -20°C for receptor binding assays.

Receptor Binding

Membrane suspensions adjusted to 50 μg protein in 400 μl of Tris-HCl buffer (25 mM, pH 7.5, with 1% BSA) are incubated for 10 min with 50 μl peptide solution (Tris-HCl buffer) and 50 μl of [^{125}I]glucagon (10^6 cpm, 25 fmol). The samples are then filtered through Oxoid filters and washed three times with 1 ml of Tris-HCl buffer. The radioactivity retained in the filters is counted by a γ -counter.

Adenylate Cyclase Assay

The assay is carried out with a membrane suspension containing 25–30 μg protein in a volume of 0.1 ml of 25 mM Tris/HCl (pH 7.5) containing 1% BSA, 1 mM ATP with 4×10^6 cpm [α - ^{32}P]ATP, 5 mM MgCl_2 , 1 nM cAMP containing 10,000 cpm of [^3H]cAMP, 10 mM GTP, 20 mM phosphocreatine and 0.72 mg/ml creatinine phosphokinase (100 U/ml). Assays are run in triplicate.

EVALUATION

Results are expressed as the percentage inhibition of [^{125}I]glucagon specific binding for receptor binding assays. For adenylate cyclase assays the results are expressed as percent potency relative to the maximal stimulation by glucagon which is defined as 100%.

MODIFICATIONS OF THE METHOD

Azizeh and coworkers (1995, 1997) synthesized and tested a glucagon antagonist and multiple replacement

analogs of glucagon by adenylate cyclase assay according to Lin and coworkers (1975) and receptor binding assay according to Wright and Rodbell (1979).

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lon et al. 1993) glucagon-like peptide1 (GLP-1) receptor has been achieved. The sequence of glucagon-like peptide-1 (7–36)amide is completely conserved in all mammalian species studied, implying that it plays a critical physiological role. Intracerebroventricular administration of GLP-1 powerfully inhibits feeding in fasted rats, which is blocked by the GLP-1-receptor antagonist exendin(9–39) (Turton et al. 1996; Meurer et al. 1999).

Peptides isolated from reptile venoms, such as exendin-4, were found to have similar activity as glucagon-like peptide-1(7–36)amide (Göke et al. 1993b; Adelhorst et al. 1994; Schepp et al. 1994). Gedulin et al. (2005) found that exendin-4 improves insulin sensitivity and β -cell mass in insulin-resistant obese *fafa* Zucker rats independent of glycemia and body weight. Shechter et al. (2003) described [2-sulfo-9-fluorenylmethoxycarbonyl]₃-exendin-4 as a long-acting glucose-lowering prodrug. Analogs and antagonists of glucagon-like peptide-1(7–36)-amide have been synthesized and evaluated in pharmacological experiments (Watanabe et al. 1994, Hjorth and Schwartz 1996, Montrose-Rafizadeh et al. 1997).

Besides insulin release from perfused pancreas (see K.6.6), the receptor binding according to Göke and Conlon (1988) on rat insulinoma-derived cells (RINm5F cells), the cAMP formation and the insulin release were studied (Göke et al. 1989a, b).

PROCEDURE

Binding Studies with RINm5F Cells

The RINm5F cell line is derived from a radiation-induced insulin-producing rat tumor (Gazdar et al. 1980). The RINm5F cells are grown in plastic culture bottles (Praz et al. 1983). They are detached from the surface of the bottles before the experiment using phosphate-buffered saline (PBS, 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3) containing 0.7 mM EDTA, and centrifuged (100 × g, 5 min). The pelleted cells are resuspended in an incubation buffer (2.5 mM Tris-HCl, 120 mM NaCl, 1.2 mM MgSO₄, 1.5 mM KCl, and 15 mM CH₃COONa, pH 7.4) containing 1% human serum albumin, 0.1% bacitracin and 1 mM EDTA. Approximately 3 × 10⁶ cells/tube are incubated for 5 min at 37°C, followed by the addition of unlabeled peptide (final concentration range from 10 pmol to 1 mmol) and radiolabeled tracer (approximately 40,000 c.p.m.). Iodination of the glucagon-like peptide-1(7–36)amide is carried out using the lactoperoxidase method. The total volume of incubation is 0.3 ml. After incubation for 60 min, aliquots (200 μ l) of the cell sus-

K.4.4

Glucagon-Like Peptide I

PURPOSE AND RATIONALE

Several intestinal peptides have been described to have insulinotropic or incretin activity, e. g. GIP (gastric inhibitory peptide, glucose-dependent insulin releasing peptide) (Creutzfeldt 1985, Baer and Dupré 1989, Volz et al. 1995). More recently, glucagon-like peptide-1 (7–37) or the (7–36)amide is described as a new incretin (Kreymann et al. 1987, Fehmann et al. 1989, 1990, 1991a, 1992, 1995, Holz et al. 1993). The insulinotropic activity has been confirmed in diabetic and non diabetic subjects (Gutniak 1992, Nathan et al. 1992, Nauck et al. 1993, Ørskov 1993). The insulin stimulatory effect of glucagon-like peptide-1(7–36)amide is glucose-dependent (Göke et al. 1993a). The peptide does not only stimulate insulin release but also inhibits glucagon secretion (Komatsu et al. 1989). Cloning and functional expression of the rat (Thorens 1992) and human (Dil-

pensions are centrifuged (11,500 × g, 2 min) through an oil layer (dibutylphthalate/dinonylphthalate, 10/4, v/v). Cell-surface associated radioactivity in the pellet is counted using a γ -counter (e. g. Gamma 5500, Beckman).

Determination of cAMP Production in RINm5F Cells

Approximately 1×10^6 cells in 0.45 ml buffer (113 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 10 mM HEPES, 2.5 mM CaCl_2 and 1.2 mM MgSO_4 , pH 7.4) containing 1% human serum albumin are pre-incubated for 10 min at 37°C, and then incubated for 10 min after the addition of 2 μl 3-isobutyl-1-methylxanthine (IBMX, 50 mM) in order to prevent the breakdown of cAMP. The reaction is then started by the addition of 50 μl of a peptide solution dissolved in the above buffer (final concentration range from 10 pM to 1 μM). After incubation for 10 min at 37°C, the reaction is stopped by the addition of 200 μl 12% trichloroacetic acid (TCA). The reaction mixture is sonicated for 30 s at 25 W (e. g., Heat system, Ultrasonics) and centrifuged (11,500 × g, 2 min). 25 μl of 1 M HCl is added to 0.5 ml supernatant. TCA dissolved in the supernatant is removed by diethyl ether (3 × 1 ml) and the resulting supernatant is stored at -40°C until cAMP assays being performed by use of a RIA kit.

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K.4.5

Insulin-Like Growth Factors

PURPOSE AND RATIONALE

Three different lines of research have led to the discovery of insulin-like growth factors (Froesch et al. 1985): Growth hormone does not stimulate growth processes by itself but induces factors, named sulfation factors or somatomedins, that mediate the message of growth hormone (Salmon and Daughaday 1957, Nevo 1982, Laron 1999). Pierson and Temin (1972) extracted factors from serum with multiplication-stimulating activity (MSA) for chicken fibroblasts in the cell culture and with non-suppressible insulin-like activity. Serum exerts insulin-like effects on insulin target tissues such as muscle and adipose tissues to a much greater extent than it can be expected on the basis of the insulin content in serum. The activity of factors other than insulin in the epididymal fat pad assay was proven by the persistence of serum insulin-like activity after pancreatectomy (Steinke et al. 1962). Since this activity could not be suppressed by insulin antibodies, the factors were called ‘non-suppressible insulin-like activity’ or NSILA.

They were finally identified as insulin-like growth factors I and II (Rinderknecht and Humbel 1978a, b) structurally related to insulin. These polypeptide hormones are present in serum in high concentrations but are bound to specific carrier proteins (Zapf et al. 1975). Most of IGF and also the IGF-binding protein are synthesized and secreted, but apparently not stored by the liver (Schwander et al. 1983, Sara and Hall 1990).

Insulin-like growth factor 1 (IGF-1) is a polypeptide of 70 amino acids, whose function of is more or less clarified (Cascieri et al. 1988, Moxley et al. 1990, Salamon et al. 1991, Schmitz et al. 1991, Vikman et al. 1989, Laron 1999). The metabolic activity of IGF-1 is regulated by at least 6 IGF-binding proteins, the most important being IGFBP-3. The effects of growth hormone on protein metabolism are mediated by IGF-1,

whereas these 2 hormones are antagonistic in their effects on insulin and some aspects of lipid metabolism. IGF-1 has been shown to improve glycemic control and to reduce insulin requirements in both IDDM and NIDDM (Simpson et al. 1998). One important clinical use is replacement therapy in primary IGF-1 deficiency, such as Laron syndrome, which is characterized by dwarfism due to primary GH resistance or insensitivity (Laron 1999). IGF-1 improves bone healing *in vivo* (Kobayashi et al. 1996). A truncated variant of human IGF-1 with the tripeptide Gly-Pro-Glu absent from the N-terminus, has been isolated from bovine colostrum, human brain and porcine uterus which is about 10-fold more potent than IGF-1 at stimulating hypertrophy and proliferation of cultured cells (Ballard et al. 1999).

The function of insulin-like growth factor II is less defined (Roth 1988). IGF-II has an insulin-like effect *in vitro* and *in vivo* (Shizume et al. 1996, Burvin et al. 1998). IGF-II, administered acutely, affects glucose homeostasis in a manner very similar to insulin, probably via the insulin receptors, although with significantly lower potency.

Two subtypes of IGF receptors exist which are different from the insulin receptor and different in their affinity for IGF I and IGF II (Rechler 1985; Verspohl et al. 1988). They belong to the insulin receptor family of receptor tyrosine kinases (Ullrich and Schlessinger 1990, Schlessinger and Ullrich 1992, Fantl et al. 1993). Although insulin and IGF bind with high affinity to their own specific receptors, each can also bind to the heterologous receptor with reduced binding affinity (DeMeys 1994).

With recombinant DNA technology insulin analogs with modified amino acid sequence can be produced which may bind differently to the insulin and IGF receptor resulting in different biological activities (Drejer 1992, Schäffer 1993). It has been speculated whether the unexpected carcinogenic effect of an insulin analog (Dideriksen et al. 1992) is related to the mitogenic effect in mouse NIH 3T3 fibroblasts which express IGF receptors but not insulin receptors (Gammeltoft and Drejer 1991). Biosynthesis of 10 kDa and 7.5 kDa insulin-like growth factor II in a human rhabdomyosarcoma cell line has been described by Nielsen et al. (1993).

For new insulin analogs the kinetics of association and dissociation to the receptors should be studied and *in vivo* metabolic activity should be compared with insulin and IGF I.

The *in vivo* metabolic action of insulin-like growth factor I can be compared with insulin in adult rats

using the following methods (Schmitz et al. 1991): Dose-dependence and time-dependence of blood sugar decrease after intravenous injection, antilipolytic effect (decrease of non-esterified fatty acids) after i.v. injection, stimulation of glucose disposal during euglycemic clamping after intravenous infusion. Furthermore, all other assays described for insulin can be applied. Gazzano-Santoro and coworkers (1998) described a cell-based potency assay for insulin-like growth factor-1.

PROCEDURE

Cells from the human cell line HU-3, established from the bone marrow of a patient with acute megakaryoblastic leukemia, are adapted to grow in the presence of human thrombopoietin. Removal of thrombopoietin results in decreased proliferation and rapid loss of viability. Cells are cultured in RPMI-1640 medium with 2% heat-inactivated human serum, 2 mM glutamine, 10 mM HEPES (pH 7.2) and 5 ng/ml thrombopoietin in culture flasks. They are grown in suspension at 37°C in a humidified 5% CO₂ incubator and are routinely subcultured every two or three days when densities reach 0.8–1.5 × 10⁶ cells/ml.

The cell growth assay is performed under serum-free conditions in assay medium consisting of RPMI-1640 supplemented with 0.1% BSA, 10 mM HEPES (pH 7.2) and 50 µg/ml gentamycin. Cells are washed twice in the assay medium and resuspended at a density of 0.25 × 10⁶ cells/ml. In a typical assay, 100 µl of a cell suspension (25,000 cells/well) and 100 µl of IGF-1 at varying concentrations are added to flat bottomed 96-well tissue culture plates at 37°C and 5% CO₂ and cultured for 2 days. 40 µl of Alamar Blue™ (undiluted) are then added and the incubation continued for 7–24 h. The plates are allowed to cool to room temperature for 10 min on a shaker and the fluorescence is read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm.

EVALUATION

Results, expressed as relative fluorescence units, are plotted against IGF-1 concentrations using a 4-parameter curve-fitting program. Test compounds are compared with the standard.

MODIFICATIONS OF THE METHOD

Boge and coworkers (1994) described an enzyme immunoreceptor assay for the quantitation of insulin-like growth factor-1 and insulin receptors in bovine muscle tissue.

Hodgson and coworkers (1995) tested mutations at positions 11 and 60 of insulin-like growth factor-1 using (a) quantification of affinities for the human insulin receptor overexpressed on NIH 3T3 cells, (b) quantification of affinities for the type I IGF receptor via competition for binding to a monolayer of MDA-MB-231 cells, (c) promotion of the *in vitro* mitogenesis of growth-arrested MCF-7 cells in the presence of 17- β -oestradiol, and (d) a competition assay for binding to IGF-binding proteins secreted by MCF-7 cells.

In order to investigate the influence of insulin-like growth factor-1 and IGF-binding protein-1 on wound healing, Lee and coworkers (1996) measured the contraction of collagen gels with embedded fibroblasts.

Ernst and White (1996) studied the hormonal regulation of IGF-binding protein-2 expression in C₂C₁₂ myoblasts.

Frystyk and Baxter (1998) described a competitive assay for rat insulin-like growth factor (IGF)-binding protein-3 (rIGFBP-3) based on the ability of IGFBP-3 to form a ternary complex with the acid labile subunit (ALS) in the presence of IGF. Human ALS was bound to test tubes pre-coated with anti-human ALS antibody. The assay depends on a competition between a covalent complex of ¹²⁵I-hIGF-I and hIGFBP-3, added as tracer, and hIGFBP-3 or rIGFBP-3 in standards or test samples, for binding to the immobilized hALS. Purified natural hIGFBP-3 served as standard.

Damon and coworkers (1997) used the C₂ skeletal myogenic cell line to characterize an insulin-like growth factor-binding protein, named Mac25/IGFBP-7.

To explore the possible relationship between insulin-like growth factor I and diabetic retinopathy, Naruse and coworkers (1996) examined the effects of glucose on IGF-I stimulated thymidine incorporation into DNA and IGF-I binding in cultured bovine retinal pericytes.

Jonsson and coworkers (1997) used a fluorometric proliferation assay with AlamarBlue to study the proliferative capacity of isolated human osteoblasts which were dose-dependently stimulated by IGF-I.

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K.4.6 Amylin

GENERAL CONSIDERATIONS

Amylin, also named islet amyloid polypeptide, is a pancreatic islet peptide consisting of 37 amino acids with a role in the maintenance of glucose homeostasis. The peptide is predominantly present in the β -cells of the pancreas and to a lesser extent in the gastrointestinal tract and in the nervous system, where amylin mRNA is also present along with specific binding sites.

Amylin has structural and functional relationships to two other messenger proteins, calcitonin and calcitonin gene-related peptide (Rink et al. 1993, Pittner et al. 1994, van Rossum et al. 1997, Wimalawansa 1996).

Amylin inhibits food intake in rodents when given centrally as well as peripherally (Morley et al. 1997, Lutz et al. 1998). Bhavsar and coworkers (1998) found a synergy between amylin and cholecystokinin for inhibition of food intake in mice.

Amylin reduces insulin-stimulated incorporation of glucose into glycogen in skeletal muscle. Young and coworkers (1992) found that increasing concentrations of amylin progressively depressed the maximal insulin-stimulated radioglucose incorporation into soleus muscle glycogen.

Castle and coworkers (1998) reported an inhibition of 3-O-methyl-D-glucose transport in perfused rat hindlimb muscle under hyperinsulinemic conditions.

Bryer-Ash and coworkers (1995) found that amylin-mediated reduction in insulin sensitivity corresponds to reduced insulin receptor kinase activity in the rat *in vivo*.

Transgenic mice expressing human islet amyloid polypeptide develop diabetes mellitus by 8 weeks of age, which is associated with selective β -cell death and impaired insulin secretion (Janson et al. 1996).

Mulder and coworkers (2000) showed that amylin-deficient mice develop a more severe form of alloxan-induced diabetes.

In single β -cells exhibiting normal glucose sensing, amylin causes membrane hyperpolarization, increases net outward current and reductions in insulin secretion. In contrast, in cells with abnormal glucose sensing (e. g., from *db/db* diabetic mice) amylin has no effect on electrical activity or secretion (Wagoner et al. 1993).

Amylin suppressed the cAMP generation induced by glucagon-like peptide 1 (GLP-1) in RINm5F cells dose-dependently (Göke et al. 1993).

Amylin, also named human islet amyloid polypeptide (hIAPP) according to van Hulst and coworkers (1997), may be implicated in the pathogenesis of pancreatic islet amyloid formation and type 2 diabetes mellitus (Wimalawansa 1997). Van Hulst and coworkers (1997) used transgenic mice for the study of (patho)physiological roles of hIAPP *in vivo*. Leckstrom and coworkers (1997) studied plasma levels and immunoreactivity of insulin and islet amyloid polypeptide in *Psammomys obesus* (sand rat) under low-energy and high-energy diet.

Amylin given peripherally or centrally inhibits acid gastric secretion in a dose dependent manner and has a protective effect against indomethacin- or ethanol-induced ulcers only when injected centrally. Subcutaneous or central injection of amylin produces a dose-dependent inhibition of gastric emptying, which may contribute to the activity of amylin in the regulation of carbohydrate absorption. In addition, amylin inhibits food intake both when injected peripherally or centrally. Amylin is considered to take part in the rapid endocrine response during digestion to maintain euglycemia (Guidobono 1998).

Amylin slows the rate of gastric emptying in spontaneously diabetic BB/Wistar rats (Macdonald 1997) and inhibits gastric acid secretion in rats (Rossowski et al. 1997).

Guidobono and coworkers (1997) reported a cytoprotective effect of amylin against indomethacin- and ethanol-induced ulcers when given intracerebroventricularly, but not when given subcutaneously.

Clementi and coworkers (1997) found a protective, dose-dependent effect of amylin against reserpine- and serotonin-induced gastric damage in rats, however, no anti-ulcer effect after pylorus ligation.

The search for a superior compound with the biological actions of human amylin resulted in the identification of [Pro^{25,28,29}]human amylin, assigned the USAN name pramlintide. Young and coworkers (1996) compared this compound with human and rat amylin in various pharmacological tests, such as receptor assays (binding to rat nucleus membranes, amylin receptors; binding to membranes from SK-N-MC cells, CGRP receptors; binding to membranes from T46D cells, calcitonin receptors), gastric emptying in rats, plasma glucose after oral glucose gavage. When studying glycogen metabolism in the isolated soleus muscle of rats, pramlintide was slightly more potent than human amylin, but not different from rat amylin in inhibiting insulin-stimulated incorporation of [U-¹⁴C]glucose into muscle glycogen. After intravenous infusion of amylin or pramlintide, a dose-

dependent increase of plasma glucose, plasma lactate and ionized calcium was found. Mean arterial blood pressure decreased with higher doses.

Clementi and coworkers (1995) found an anti-inflammatory activity of amylin in mouse ear edema induced by Croton oil and acetic acid-induced peritonitis in the rat, but not in serotonin-induced rat paw edema and plasma protein extravasation induced by dextran in rat skin.

Bell and coworkers (1995), Bell and McDermont (1995) reported that amylin has an hypertrophic effect in rat ventricular cardiomyocytes and exerts the contractile response via CGRP₁-preferring receptors.

In anesthetized rats, amylin increased after intravenous infusion urine flow, sodium excretion, glomerular filtration rate and renal plasma flow (Vine et al. 1998).

Clementi and coworkers (1996) reported that intracerebroventricularly injected amylin induced in rats a dose-dependent decrease of locomotor activity without affecting grooming and sniffing.

Amylin dose-dependently stimulated cell proliferation of human osteoblast like (hOB) cells and increased osteocalcin production (Villa et al. 1997). Cornish and coworkers (1998) found in adult male mice after daily subcutaneous injection of amylin for 4 weeks an increase of histomorphometric indices of bone formation and a reduction of bone resorption. Muff and coworkers (1995) and Poyner (1997) identified, characterized and cloned the receptors for calcitonin, calcitonin-gene-related peptide and amylin.

PURPOSE AND RATIONALE

Binding sites with high affinity for amylin are present in several brain regions, with the nucleus accumbens and surrounding tissue containing more than twice as many binding sites as any other regions (Beaumont et al. 1993).

PROCEDURE

Membrane Preparation

Membranes are prepared from male Sprague-Dawley rats (150–200 g). Following decapitation, the basal forebrain regions (nucleus accumbens) are removed to PBS (pH 7.4) at 4°C. The tissues are weighted, then placed in 10 ml/g tissue of ice-cold 20 mM HEPES/KOH (pH 7.4) and homogenized with a Polytron (10 s at setting 4). An additional 30 ml of cold HEPES/KOH is added, and the homogenate centrifuged (48,000 × g, 15 min). After discarding the supernatant fluid, membrane pellets are resuspended by homogenization in 40 ml of fresh HEPES/KOH and

centrifuged as before. Membranes are washed again by homogenization in buffer and centrifugation. The final membrane pellet is resuspended in a volume of 20 mM HEPES/KOH containing 0.2 mM PMSF added immediately before use from a stock 0.2 M solution in ethanol. A volume of buffer is used to yield a concentration of about 80 mg original tissue/ml. Membranes are kept frozen at -80°C until use.

Binding Assay

Membranes from 4 mg of original wet weight of tissue are incubated with ^{125}I -BH-amylin (rat amylin, BH-labeled at the amino-terminal lysine, (Amersham Corp.) in 20 mM HEPES/KOH (pH 7.4), containing 0.5 mg/ml bacitracin, 0.5 mg/ml BSA and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), for 60 min at 23°C . Incubations are carried out in duplicate tubes and are started by addition of membranes. Incubations are terminated by filtration through glass fiber filters that have been presoaked in 0.3% polyethylene-imine, followed by washing with 15 ml of cold PBS.

EVALUATION

Competition curves are generated by measuring binding of 13 pM ^{125}I -BH-amylin in the presence of 10^{-11} to 10^{-6} unlabeled peptide. Data are fitted to a four-parameter logistic equation to derive half-maximal inhibitory concentrations (IC_{50} values) and slope factors (Hill coefficients).

MODIFICATIONS OF THE METHOD

Sheriff and coworkers (1992) characterized amylin binding sites in the human hepatoblastoma cell line, HepG2. Perry and coworkers (1997) studied amylin and calcitonin receptor binding in the mouse α -thyroid-stimulating hormone thyrotroph cell line.

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K.5 Insulin Target Tissues and Cells

K.5.1 Adipose Tissue and Adipocytes

GENERAL CONSIDERATIONS

Rodent adipose tissue and cells represent the targets exhibiting the most prominent insulin sensitivity (i. e. lowest EC/IC₅₀) and responsiveness (i. e. highest fold stimulation/inhibition above basal) of the relevant insulin signaling cascades (e. g. insulin receptor activation) and metabolic end effector systems (e. g. lipolysis) in comparison to liver (e. g. gluconeogenesis) and muscle cells (e. g. glucose transport). This might be based in part on technical advantages of the adipose tissue/adipocyte preparation in comparison to that of muscle/myocytes. But more likely it reflects the exquisite physiological role of the adipose tissue in the regulation and coordination of glucose and lipid metabolism, i. e. insulin stimulation of lipid synthe-

sis (lipogenesis) and insulin inhibition of lipolysis. On basis of their relatively easy technical preparation, functional adipose tissue fragments (epididymal fat pads) and primary adipocytes (isolated epididymal adipocytes) from rats as well as adipocyte cell lines derived from mice (3T3-L1, F442A) are the first choice for the development of robust and reliable cell/tissue-based assay systems for insulin-like activity.

K.5.1.1 Epididymal Fat Pads of Rats

GENERAL CONSIDERATIONS

Insulin-like activity can be measured by the uptake of glucose into fat cells, which at low extracellular glucose concentrations (see K.6.1.6) represents the rate-limiting step for the conversion of glucose in and its storage as lipids, or for the oxidative (glycolysis plus citric acid cycle) and non-oxidative (glycogen synthesis) metabolism of glucose. Adipose tissue from the epididymal fat pads from rodents has been found to be very suitable for the analysis of non-oxidative and oxidative glucose metabolism. Early studies (Beigelman 1959, Steelman et al. 1961) determined the difference of glucose concentration in the medium after incubation of pieces of epididymal rat adipose tissue for the direct determination of glucose uptake (encompassing non-oxidative and oxidative glucose metabolism) or measured oxygen consumption in Warburg vessels (Ball and Merrill 1961, Froesch et al. 1963) or the release of radiolabeled ¹⁴CO₂ from [¹⁴C]glucose after its trapping and counting (Martin et al. 1958, Slater et al. 1961, Gliemann 1965a and 1967b, 1972) monitoring glucose oxidation, exclusively. The latter principle was used by Martin and coworkers (1958) using epididymal fat pads from the rat. This method has been used by various authors (Humbel 1959, Ditschuneit et al. 1959) and described in detail by Renold and coworkers (1960) and Siess and coworkers (1965). Sönksen and coworkers (1965) found a close correlation between “suppressible” insulin-like activity measured by the fat pad method and insulin concentration determined by immunoassay. Ball and Merrill (1961) used the manometric measurement of the net gas exchange of rat adipose tissue to quantitate small amounts of insulin. A survey of the biological assays of insulin-like serum activity was given by Faulhaber and Ditschuneit (1975).

The use of serum from pancreatectomized animals revealed the presence of factors apparently exerting insulin-like activity other than insulin (Steinke et al. 1962, 1965). Subsequently, the nature of these insulin-

like activities has been characterized (see K.6.3.6.4), but so far they escaped their unequivocal characterization and molecular identification, presumably due to their structural heterogeneity and low abundance.

PURPOSE AND RATIONALE

The epididymal fat pad assay, originally developed as bioassay of insulin-like activity in serum samples, can also be used for measuring activity of synthetic insulin derivatives and analogs as well as for the evaluation of peripheral insulin-like effects of antidiabetic compounds, such as sulfonylureas and biguanides, which have long been assumed to exert their blood glucose decreasing activity exclusively *via* stimulation of insulin release and improvement of peripheral insulin sensitivity, respectively. However, in the subsequent decades this tissue-based system has been replaced by cell-based assays (see below) which for most applications offer the advantages of higher sensitivity, accuracy, reproducibility and test capacity (throughput) despite lower expenditure in the number of animals (*per data point*) required.

K.5.1.2

Primary Rat Adipocytes

PURPOSE AND RATIONALE

The worldwide dramatic increase in obesity with its associated health problems and the identification of adipocyte-secreted proteins that apparently have major impact on the regulation of whole-body glucose, lipid and energy metabolism have generated major interest in adipocyte biology. Adipocytes are the primary storage site for energy in vertebrate animals. During fasting, adipocytes release energy-rich molecules that provide metabolic fuels to other tissues. Adipocytes also secrete hormones that orchestrate the storage, release, and oxidation of energy-rich molecules throughout the body and that control behavior, including feeding and appetite. Primary adipocytes maintain a large dynamic pool of triacylglycerol (TAG) and express a specific set of proteins to maintain circulating metabolic fuel levels. Primary adipocytes and adipose tissue have been used to study basic adipocyte biology.

Rodbell (1964) and Gliemann (1965, 1967a and b) used primary adipocytes isolated by digestion of epididymal fat pads from rats with collagenase. The conversion of (3- ^3H) glucose into total adipocyte TAG has been used as a very sensitive parameter in an improved assay for insulin-like activity by Moody and coworkers (1974) which during the subsequent decades turned out to represent the golden standard

for assays for insulin-like activity. Alternatively to primary rat adipocytes, Etherton and Chung (1981) and Etherton and Walker (1982) characterized the insulin sensitivity of isolated swine adipocytes.

Preparation of Rat Adipocytes

Male Wistar or Sprague-Dawley rats weighing 140–200 g are sacrificed and both epididymal fat pads are removed under sterile conditions. The fat pads are cut into pieces and incubated for 20 min at 37°C with 1 mg/ml collagenase (e.g. type CLS II, Worthington, NJ) in Krebs-Ringer HEPES bicarbonate buffer, KRHB, (25 mM HEPES/KOH, pH 7.4, 0.1 mM glucose, 1% wt/vol BSA). The cell suspension is filtered through a 100- μm nylon screen and washed 3 times by flotation (accumulation of a thin cell layer on top of the medium after centrifugation (1,000 \times g, 1 min, swing-out rotor) with KRHB lacking glucose and finally suspended in the same solution. The suspension is adjusted to a final titer of 3.5×10^5 cells/ml.

Stimulation of Rat Adipocytes with Insulin

Adipocytes are suspended in buffer S (Dulbecco's minimal essential medium ([DMEM] for primary culture to induce insulin resistance, see K.6.3.5) or KRHB (for short-term measurement of insulin-like activity) both containing 5 mM glucose, 0.5 mM sodium pyruvate, 4 mM L-glutamine, 200 nM 1-methyl-2-phenylethyladenosine, 100 $\mu\text{g/ml}$ gentamycin, 1% BSA and 25 mM HEPES/KOH, pH 7.4) at 5% cytocrit (corresponding to about 7×10^5 cells/ml). For determination of the packed cell volume, small aliquots of the cell suspension are aspirated into capillary hematocrit tubes and centrifuged for 90 s in a micro-hematocrit centrifuge in order to measure the fractional occupation of the suspension by the adipocytes (= cytocrit). A 20-ml portion of the adipocyte suspension (5% cytocrit) is added to 20 ml of buffer S containing human insulin as indicated. Incubations (20 min, 37°C) are performed under 5% CO_2 in 200-ml polyethylene vials during shaking at 110 cycles/min with a stroke length of 3.5 cm.

Permeabilization of Rat Adipocytes

Studies of intermediary metabolism and its regulation by insulin often require the use of agents that do not readily penetrate the plasma membrane of intact cells due to the size and charge of substrates (e.g. sugar phosphates, nucleotides, hydrophilic inhibitors, antibodies). Such studies would greatly benefit if the permeability of the plasma membrane to these compounds or the release of freely diffusible cytoplas-

mic molecules (e. g. metabolites, proteins) across the plasma membrane could be increased. Thus, permeabilized cells can be useful for the analysis of complicated metabolic processes as well as for the study of the insulin-like activity of agents which do not readily pass the plasma membrane of adipocytes including the elucidation of the molecular mechanism(s) of the insulin(-like) action induced or inhibited by a certain compound/drug. For this, it may be useful to block known signal transduction or metabolic pathways in adipocytes by introducing specific inhibitory antibodies (against key signaling proteins/metabolic enzymes) or peptide substrates (competing for the endogenous protein substrate; e. g. synthetic peptide as kinase substrate) prior to stimulation of the adipocytes with the compound/drug. In conclusion, permeabilized cells should be looked upon as a method that rapidly makes the interior of the cells accessible (for a review see Declercq and Baes 2000).

In general, the widely used methods of short incubation under hypoxic conditions (Gordon et al. 1985) and electroporation (i. e. subjecting cells to a short series of high-voltage electrical discharges permeabilize cells transiently. These cells are characterized by the formation of small protuberances (blebs) indicative of localized cell surface damage. It is conceivable that the stretched plasma membrane of such blebs act as a high-permeability region. Disappearance of blebs and restoration of normal plasma membrane impermeability is achieved again by short (15-min) incubation at 37°C. During the period of enhanced permeability small compounds can be introduced. Upon alternative physical treatment, such as osmotic shock and brief freezing/thawing, the cells are also temporarily permeabilized. Such cells reseal under appropriate conditions (Lepers et al. 1990). These physical methods are probably not selective for the plasma membrane necessitating other permeabilization techniques if intact intracellular membranes are required.

In addition, intactness of intracellular organelles will also benefit from the use of permeabilizing media that are isotonic with the cell content (Bijleveld and Geelen 1987). The cholesterol-sequestering approach produces very fast holes of about 8 nm (Schulz 1990) and is most suited to rapidly measuring cellular enzyme activities as the lesions are sufficiently large to allow entry of large molecular substrates or proteins and to allow cytosolic proteins of at least 285 kDa to leave the cell (Bijleveld and Geelen 1987). Apart from transient permeabilization, all the other techniques for permeabilizing cells have severe limitations due to the fact that cellular integrity is destroyed. The degree of

loss of integrity determines the application of a particular permeabilizing procedure. The use of digitonin-permeabilized mammalian cells for measuring enzyme activities in the course of studies on lipid metabolism has been reviewed by Geelen (2005).

Electroporation of Rat Adipocytes

For the introduction of antibodies and peptides as well as compounds/drugs into isolated rat adipocytes without significant loss of cell viability and insulin sensitivity, the method of electroporation has been used successfully. For this, 0.4 ml of buffer E (4.74 mM NaCl, 118 mM KCl, 0.38 mM CaCl₂, 1 mM EGTA, 1.19 mM MgSO₄, 1.19 mM KH₂PO₄, 25 mg/ml BSA, 3 mM sodium pyruvate and 25 mM HEPES/KOH, pH 7.4) is placed in a 0.4-cm gap-width electroporation cuvette (Bio-Rad, Munich, Germany) together with the antibodies or peptides or compounds/drugs. 0.4 ml of adipocyte suspension (50% cytocrit in buffer E) is added to each cuvette and gently mixed. Electroporation is performed using a Gene Pulser Transfection Apparatus (Bio-Rad, Munich, Germany) which is set at a capacitance of 25 µF and voltage of 800 V (2 kV/cm), at 25°C for six shocks (Shibata et al. 1991, Quon et al. 1993). After the third treatment, the adipocyte suspension is gently stirred with a plastic stick and the electric polarity is reversed. The time constant of electroporation is typically 0.6 ms during the final shock. Routinely, 4 ml of adipocyte suspension (25% cytocrit) is electroporated in five cuvettes. The time required for treatment of the five cuvettes is about 3 min. After electroporation, the cells from five electroporations are pooled and transferred to 50-ml polystyrene tubes. After incubation (30 min, 37°C) in 5% CO₂/95% O₂, the cells are centrifuged (200 × g, 1 min, swing-out-rotor) and the infranant is aspirated. Thereafter the cells are washed once with 40 ml of buffer E containing 5 mM glucose and 4% BSA, suspended in 20 ml of the same buffer and then incubated (1 h, 37°C) under 5% CO₂ prior to challenge with the appropriate stimulus. Using this electroporation technology Müller and coworkers (2000) introduced into rat adipocytes inhibitory antibodies against tyrosine kinases mediating insulin-like signaling in response to stimuli different from insulin (see K.6.3.6.4).

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K.5.1.3

Insulin-Resistant Primary Rat Adipocytes

PURPOSE AND RATIONALE

Primary rat adipocytes can be maintained in culture for long-term treatment with compounds/drug candidates to study their insulin-like activity upon chronic challenge or for the induction of insulin resistance to study the insulin-sensitizing activity of compounds/drugs for up to 24 h without significant loss of viability by incubation in buffer S (see K.6.3.5) containing 5 mM glucose and 0.5 mM sodium pyruvate under 95% O₂/5% CO₂ and continuous gentle shaking with moderate loss of insulin sensitivity and responsiveness, only (Müller and Wied, 1993). The conditions can be modified for the (subsequent) induction of insulin resistance *in vitro*, which might reflect the hyperglycemic and hyperinsulinemic state promoting insulin resistance *in vivo*.

PROCEDURE

For induction of insulin resistance, primary rat adipocytes are incubated in buffer S in the presence of 25 mM glucose, 0.5 mM sodium pyruvate and 10 nM insulin for 4 to 20 h at 37°C under slow shaking. When assayed for insulin-stimulated glucose transport following a 15- to 30-min incubation period in buffer S

containing 0.5 mM glucose and sodium pyruvate but lacking insulin (which enables downregulation of the glucose transport system from the chronically insulin-stimulated to basal levels).

EVALUATION

The adipocytes made insulin-resistant *in vitro* show a right-ward shift of the concentration-response curve (decreased insulin sensitivity) and a reduced maximal glucose transport velocity at constant or only slightly elevated basal glucose transport (decreased insulin responsiveness) (see Müller and Wied 1993). During prolonged incubation in suspension culture, the adipocytes tend to increase their basal glucose uptake which might reflect elevated energy demands due to stress conditions or loss of plasma membrane integrity. It is therefore most critical to choose culture conditions which preserve the energy status of the rat adipocytes and are compatible with 5- to 6-fold stimulations of glucose transport, at least, at low glucose concentrations corresponding to the normal insulin-sensitive state. This allows monitoring of the induction of insulin resistance by culturing of the adipocytes at high glucose and insulin and its blockade by the concomitant presence of compounds/drugs which may act as insulin sensitizers. The use of 25 mM glucosamine instead of high glucose/insulin as initially proposed by Marshall and coworkers (1984; see K.6.3.5) on basis of the apparent activation of the hexosamine pathway during glucose/insulin-induced desensitization of rat adipocytes in culture is not recommended. It has meanwhile been demonstrated that intracellular accumulation of glucosamine results in depletion of cytosolic ATP and accompanying induction of the stress response (Hresko et al. 1998) causing apparent loss of insulin responsiveness which is based on massive increment of the basal glucose transport rate rather than on impairment of insulin signaling to the glucose transport system.

K.5.1.4

Cultured Mouse Adipocytes

GENERAL CONSIDERATIONS

Primary adipocytes have several limitations. They do not propagate in culture, they are difficult to transfect with DNA, they usually have a single huge TAG lipid droplet (LD) that interferes with biochemistry and microscopy, their properties vary as a result of the genetics and conditions of the animals from which they have been isolated, and the isolation procedure is tedious and introduces variation. In addition, harvesting pri-

mary adipocytes or adipose tissue from animals generally requires the euthanasia of a vertebrate animal and the expense of specialized facilities and protocols. For these reasons and likely others, cell lines have been developed that can be induced to store TAG, to express proteins that are hallmarks of primary adipocytes, exert many of the functional and physiological features of primary adipocytes and presumably to recapitulate key events in adipocyte ontogeny.

K.5.1.4.1

3T3-L1 Adipocytes

PURPOSE AND RATIONALE

Three decades ago, Kehinde and Green (1974) reported that a clonal subline of mouse 3T3 cells had a propensity to differentiate into adipocytes when in a "resting state". This 3T3-L1 cell culture model of adipogenesis has been exploited extensively to investigate the molecular mechanisms of adipocyte differentiation, lipid metabolism, insulin signaling, and glucose transport as well as to identify physiologically important adipocyte-secreted proteins (adipokines or adipocytokines), such as adiponectin, visfatin and resistin (for reviews see Banerjee and Lazar 2003, Stefan and Stumvoll 2002, McGillis 2005).

Green and Kehinde (1974) isolated from the established mouse fibroblast line 3T3 two subclonal lines that accumulate large amounts of TAG after they had undergone stimulus-induced differentiation from confluent fibroblasts *via* the state of preadipocytes to mature adipocytes. The cell line 3T3-L1 has been used extensively by many authors (Spooner et al. 1979, Frost and Lane 1985, Zuber et al. 1985, Chan et al. 1988, Clancy and Czech 1990, Wieland et al. 1990, Müller et al. 1993). For some purposes (e.g. analysis of the endogenous leptin production), the F442A mouse fibroblastic cell line has been used successfully (Zhang et al. 2002, Teta et al. 2005). Kletzien and coworkers (1992) studied the effect of pioglitazone, an insulin-sensitizing agent, on the expression of the adipocyte fatty acid-binding protein in *ob/ob* mice and 3T3 L1 cells.

PROCEDURES

Culture and Differentiation

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) are seeded in 12-well (60,000 cells/well) or 96-well (15,000 cells/well) plates and maintained in DMEM (high glucose), 10% fetal bovine serum (FBS), 5 mM L-glutamine and 2% BSA. Following three days at 90–100% conflu-

ence, differentiation is initiated by the addition of DMEM containing 10% FBS, 400 nM human insulin, 1 μ M dexamethasone and 1 mM IBMX. Three days later, the medium is replaced with DMEM, 10% FBS and 100 nM insulin. After additional two days, the medium is changed to DMEM (low glucose) and 10% FBS. The presence of putative insulin-sensitizing compounds, such as glitazones (e. g. 1–10 μ M pioglitazone) in the various differentiation media considerably may improve the degree of differentiation as has been shown in numerous studies since the introduction of these drugs into the anti-diabetic therapy. The differentiated adipocytes are used 5 to 12 d after completion of the differentiation protocol, when more than 85% of the cells expressed the adipocyte phenotype (LD accumulation as observed with “Oil Red” staining). Prior to experimentation, the cells are rinsed two times with low serum medium (DMEM containing 5 mM glucose, 0.5% BSA, 0.1% FBS, 25 mM HEPES/KOH, pH 7.4, 10 mM glutamine, 100 units/ml streptomycin/penicillin), then incubated (12 to 14 h) in this medium lacking FBS (for down-regulation of the insulin signal transduction cascade) and finally washed twice with PBS containing 2 mM sodium pyruvate prior to incubation (30 min, 37°C) with insulin or compounds/drug candidates.

K.5.1.4.2

OP9 Adipocytes

PURPOSE AND RATIONALE

The 3T3-L1 adipocyte model has significant limitations. First, from the time of initial plating, the generation of 3T3-L1 adipocytes from preadipocytes requires at least two weeks (Student et al. 1980). Second, if 3T3-L1 cells become confluent and are further propagated or if they are passaged extensively, they no longer differentiate robustly into adipocytes. These issues make culturing of 3T3-L1 cells demanding and limit their utility in the generation of stable cell lines. Third, it is difficult to efficiently detect RNAs and proteins encoded by transiently transfected DNA in 3T3-L1 adipocytes. This limitation derives from the facts that most plasmid transfection protocols require subconfluent cells and that levels of RNAs and proteins expressed from such transfections dramatically decline before 3T3-L1 cells become adipocytes. Finally, because the 3T3-L1 cell line originated from a single clone and thus has clone-specific traits, it fails to recapitulate the primary cells it models. Thus, an alternative, tractable adipocyte model system would be desirable. A new adipocyte cell culture model, OP9

mouse stromal cells, has been characterized recently that provides a tractable alternative system for studies of adipocyte biology (Wolins et al. 2006). The OP9 cell line was established from the calvaria of newborn mice genetically deficient in functional macrophage colony-stimulating factor (M-CSF) (Nakano et al. 1994).

PROCEDURE

Propagation and Differentiation

OP9 cells are available from the American Type Culture Collection (ATCC, catalog no. CRL-2749), however, have to be tested and selected for those capable of consistent differentiation into adipocytes. OP9 cells are grown in OP9 propagation medium (α -MEM, 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin). The optimal differentiation potential of OP9 cells is achieved when cells are replated every three days at a density of at least 7,000 cells/cm². OP9 cells grown in this manner are flat with broad cell processes. In contrast, OP9 cells maintained at low cell density adopt a spindly morphology and differentiate into adipocytes poorly. Three methods can be used to differentiate OP9 preadipocytes into adipocytes.

Serum Replacement Method (SR)

OP9 cells are grown to confluence and then cultured for two additional days in either OP9 or 3T3-L1 propagation medium as described above. The cells are then cultured up to 4 more days in serum replacement medium (α -MEM, 15% KnockOut™ SR [Invitrogen], 100 U/ml penicillin, and 100 μ g/ml streptomycin). For the purpose of studying effects of insulin/compounds/drugs, it is important to change the SR medium to OP9 propagation medium after day 2 of differentiation, because KnockOut™ SR contains very high concentrations of insulin (final concentration of 1.7 μ M) and 5 ng/ml bone morphogenic protein 4 (BMP-4; R&D Systems). However, BMP-4 makes no significant difference in adipocyte differentiation, so all experiments can be performed without BMP-4 with the exception of the assay for insulin-stimulated glucose uptake.

Insulin/Oleate Method (IO)

OP9 cells are plated at 5,000 cells/cm². When cells adhered to the plate, the OP9 propagation medium is replaced with insulin/oleate medium (α -MEM, 0.2% FBS, 175 nM insulin, 900 μ M oleate bound to BSA [5.5/1 molar ratio] prepared according to Wolins and coworkers (2005); 100 U/ml penicillin, and 100 μ g/ml streptomycin).

Adipogenic Cocktail Method (AC)

This method is very similar to that used for 3T3-L1 cells (see K.5.1.4.1 and Student et al. 1980). Cells are grown to confluence and then cultured for two additional days in 3T3-L1 adipocyte medium (DMEM, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). Cells are then cultured for two days in DM1 (DMEM, 10% FBS, 175 nM insulin, 0.25 µM dexamethasone, 0.5 mM IBMX, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). The cells are cultured for two additional days in DM2 (DMEM, 10% FBS, 175 nM insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). Differentiated OP9 cells are maintained in OP9 propagation medium.

EVALUATION

OP9 mouse stromal cells represent a new model of rodent adipocytes that will be useful for further studies of the mechanisms of adipocyte differentiation and of mature adipocyte function (Wolins et al. 2006). When OP9 cells are given any one of three adipogenic stimuli, they rapidly accumulate TAG to form numerous, large LD, exert adipocyte morphology, express adipocyte late marker proteins, including glucose transporter 4 (GLUT4), adiponectin, perilipin, and S3-12 and exhibit robust insulin-stimulated glucose transport. OP9 cells can differentiate into adipocytes within two days. This rapid rate of differentiation allows for the detection of transiently expressed proteins in mature OP9 adipocytes. Adipogenesis in OP9 cells involves the master transcriptional regulator of adipocyte differentiation, peroxisome proliferator-activated receptor (PPAR- γ). OP9 cells are late preadipocytes in that, before the addition of adipogenic stimuli, they express the adipocyte proteins CCAAT/enhancer binding proteins α and β , PPAR- γ , sterol-regulatory element binding protein-1, S3-12, and perilipin. OP9 differentiation is not diminished by maintenance in culture at high cell density or by long periods in continuous culture, thereby facilitating the generation of stable cell lines that retain adipogenic potential.

Several practical features of OP9 cells make this cell line a particularly convenient model for adipocyte studies. First, OP9 adipocytes can be rapidly produced from either preconfluent cells (IO method) or confluent cells (SR and AC methods). Second, in contrast to 3T3-L1 cells, OP9 cells can be maintained at high density without loss of potential to differentiate into adipocytes. Third, OP9 cells continue to differentiate well even at high passage number. These features per-

mit large numbers of OP9 cells to be conveniently maintained in culture and OP9 adipocytes to be produced for experiments within a few days.

K.5.1.5**Cultured Human Adipocytes**

Murine cell models, most notably the 3T3-L1 cell line, have been the basis for the majority of studies of lipogenesis and lipolysis including their regulation as well as of adipogenesis at the transcriptional and post-transcriptional levels. However, there is a growing concern that the regulation of lipid metabolism may differ between human and murine adipocytes. For example, the resistin gene and its secreted protein product as well as its putative role in the regulation of lipid synthesis and degradation were first identified in the 3T3-L1 cells. Subsequent *in vivo* analysis in mice demonstrated an association between resistin levels, obesity, and type II diabetes. In contrast, clinical studies do not demonstrate a comparable association between serum resistin levels, obesity, and insulin resistance in non-obese and obese human subjects. Likewise the expression of the agouti gene in adipose tissue and its involvement in lipolysis regulation differs between man and mouse. These discrepancies argue for the increased use of human preadipocyte cell models in exploratory research and drug discovery relating to obesity and type II diabetes. Recently, the culturing of human adipocytes has been introduced successfully. The dramatically increasing incidence of diabetes and obesity in the industrialized countries immediately argues for the need of *in vitro* studies with human adipocytes, which justifies a more detailed evaluation of their generation and characterization. Two different sources for differentiated human adipocytes are available so far which differ in their accessibility for daily use and reflection of the primary adipocyte phenotype.

K.5.1.5.1**Adipocytes Derived from Human Adipose-Derived Adult Stem (ADAS) Cells****PURPOSE AND RATIONALE**

ADAS cells can be reproducibly isolated from liposuction aspirates through a procedure involving collagenase digestion, differential centrifugation, and expansion in culture. A single milliliter of tissue yields over 400,000 cells (Aust et al. 2004). The undifferentiated human ADAS cells express a distinct immunophenotype based on flow cytometric analyses and, following

induction, produce additional adipocyte-specific proteins (Aust et al. 2004, Gronthos et al. 2001, Halvorsen et al. 2001, Sen et al. 2001, Zuk et al. 2002). The human ADAS cells display multipotentiality with the capability of differentiating along the adipocyte, chondrocyte, myogenic, neuronal, and osteoblast lineages (Aust et al. 2004, Zuk et al. 2002). In the presence of dexamethasone, insulin, IBMX, and a thiazolidinedione, the undifferentiated human ADAS cells undergo adipogenesis. Between 30 and 80% of the cells, based on flow cytometric methods, accumulate LD, which can be stained for neutral lipids with the “Oil Red” dye.

PROCEDURE

LD proteomics Proteomic procedures for the analysis of human (ADAS) and rodent fat cells have been used by DeLany and coworkers (2005), Lee and coworkers (2006), Chen and coworkers (2005) and Brasaemle and coworkers (2004). For the evaluation of ADAS cells (DeLany et al. 2005), liposuction aspirates from subcutaneous adipose tissue sites can be obtained from male and female subjects (30–40 years, 25–30 BMI) undergoing elective procedures in local plastic surgical offices. Tissues are washed three to four times with PBS and suspended in an equal volume of PBS supplemented with 1% bovine serum and 0.1% collagenase type I prewarmed to 37°C. The tissue is placed in a shaking water bath at 37°C with continuous agitation for 60 min and centrifuged (300 × g, 5 min, room temperature). The supernatant is removed, and the pelleted stromal vascular fraction (SVF) is resuspended in Stromal Medium (DMEM/F-12 Ham’s, 10% FBS, antibiotics/antimycotics) and plated at a density of 0.156 ml of tissue digest/cm² of surface area in T225 flasks using Stromal Medium for expansion and culture according to the procedure of Hauner and coworkers (1989). This initial passage of the primary cell culture is referred to as “Passage 0.” Following the first 48 h of incubation at 37°C at 5% CO₂, the cultures are washed with PBS and maintained in Stromal Medium until they achieve 100% confluence (mean cell density of ~30,000 cells/cm² after 4 d in culture). The cells are passaged by trypsin/EDTA digestion and seeded at a density of 30,000 cells/cm² (“Passage 1”) on 10-cm plates.

One day after seeding, plates are either harvested for protein (day 0), or the medium is replaced with an adipogenic differentiation (AD) medium composed of DMEM/F-12 with 3% FBS, 33 μM biotin, 17 μM pantothenate, 1 μM bovine insulin, 1 μM dexamethasone, 0.25 mM IBMX, 5 μM rosiglitazone, and 100 units of

penicillin, 100 μg of streptomycin, 0.25 μg of Fungizone. After three days, AD medium is changed to adipocyte maintenance medium, which is identical to the AD medium except for the removal of both IBMX and rosiglitazone. Cells are fed every 3rd day and usually maintained in culture for 9 d prior to harvest.

EVALUATION

The studies published so far provide a composite profile of the proteome of undifferentiated human ADAS cells obtained from multiple donors. Overall, the results confirm the adipocyte-specific protein expression. However, not all of the proteins identified derive from ADAS cells and may be qualified as contaminants. The most striking examples are hemoglobins A and B, reported in the undifferentiated cell lysates. The presence of hemoglobin apparently reflects the cell isolation procedure. The SVF population prepared by collagenase digestion of the adipose tissue and subsequent centrifugation routinely contains erythrocytes that are washed away after a 48-h period during which the ADAS cells adhere to the plastic surface. Typically, nucleated hematopoietic cells, expressing the marker CD45, account for less than 1% of the ADAS cell population by Passage 1 according to previous flow cytometric studies (Aust et al. 2004, Gronthos et al. 2001). Nevertheless it is feasible that some hemoglobin remains bound to the adherent ADAS cell population either as intact erythrocytes or as membrane-bound protein.

Proteomic analyses of total cell lysates from murine 3T3-L1 adipocytes have identified between eight and 100 protein features by one- and two-dimensional gel electrophoresis/mass spectroscopy (Welsh et al. 2004, Wilson et al. 2003, Brasaemle et al. 2004, Choi et al. 2004). The ADAS cell proteome includes a high percentage of identical or similar proteins (DeLany et al. 2005).

K.5.1.5.2

Adipocytes Derived from Commercially Available Human Preadipocytes

PROCEDURE

Frozen human preadipocytes (PromoCell) are thawed and cultured in preadipocyte expansion medium (DMEM/F12 supplemented with 10% FBS, human epidermal growth factor (2.5 ng/ml), hTGF-β (0.25 ng/ml), bovine fibroblast growth factor (bFGF, 0.5 ng/ml), and antibiotics until the cells are confluent. For adipocyte terminal differentiation, preadipocytes are induced with differentiation medium contain-

ing DMEM/F12, 3% rabbit serum, biotin (33 μ M), pantothenate (17 μ M), human recombinant insulin (0.1 μ M), dexamethasone (1 μ M), IBMX (0.5 mM), and a peroxisome proliferator-activated receptor (PPAR) γ -ligand, such as troglitazone (3 μ M) or F-moc-L-leucine (30 μ M). After a 6-d induction period, with fresh medium added every 3rd day, the cells are fed with the same medium without IBMX or a PPAR γ -ligand (adipocyte medium) every 3–4 d for 6 additional days. Cells at passages 3–4 are routinely used for adipocyte differentiation. TAG accumulation is confirmed by staining formaldehyde-fixed cells with the neutral lipid-specific dye, Bodipy, at 1 μ g/ml. Microscopic images are obtained using an inverted microscope with illuminator and image acquisition system (Carl Zeiss, Inc., Germany).

EVALUATION

Differentiated adipocytes are fixed in formaldehyde (5%), and TAG is stained with Bodipy. After the fluorescence of each well has been read at 530 nm, the Bodipy-stained lipid is removed, and cells are permeabilized using isopropanol. Isopropanol is subsequently removed, and cellular DNA is stained with EthD-1 (2 μ M) for 30 min before measuring EthD-1 fluorescence at 620 nm. The EthD-1 readout is directly correlated with cell density between 2,000 and 40,000 cells/cm². Fluorescence units of Bodipy and EthD-1 per well are used to determine the lipid/DNA ratio.

K.5.1.6

Adipocyte–Myocyte Co-Culture

PURPOSE AND RATIONALE

There is growing evidence that paracrine/endocrine communication between adipose and muscle cells may be involved in the induction of insulin resistance during the pathogenesis of type II diabetes. This negative cross-talk, which may be mediated by soluble factors secreted by the adipocytes (e. g. adipokines) can be mimicked *in vitro* by using a co-culture based on human primary skeletal muscle and human primary adipose cells as has been recently introduced by Eckel and coworkers (Dietze et al. 2002).

PROCEDURE

Culture of Human Skeletal Muscle Cells

Satellite cells are isolated from M. rectus abdominis by enzymatic digestion with trypsin followed by a purification step with fibroblast-specific magnetic beads to

prevent contamination with fibroblasts. After two passages, the myoblasts are characterized by the manufacturer (PromoCell) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100% confluence (8 d). Primary human skeletal muscle cells of four healthy donors are usually supplied as proliferating myoblasts (5×10^5 cells). These cells are grown up to confluence in 25 cm² flasks, trypsinized, and subsequently seeded in 175 cm² flasks at a density of 1×10^6 cells. After two passages $5\text{--}7.5 \times 10^7$ cells are harvested and stored until further use as frozen aliquots containing 2×10^6 myoblasts. For an individual experiment, myoblasts are seeded in 6-well culture dishes (9.6 cm²/well) at a density of 10^5 cells per well and are cultured in α -MEM/Hams F-12 medium containing “Skeletal Muscle Cell Growth Medium Supplement Pack” to up to near confluence. The cells are then differentiated and fused by culture in α -MEM for 4 days.

Isolation and Culture of Human Adipocytes

Adipose tissue samples are obtained from the mammary fat of normal or moderate overweight women (BMI 20–30 kg/m², 20 and 50 years) undergoing surgical mammary reduction. All subjects should be healthy and have no evidence of diabetes according to routine laboratory tests. Adipose tissue samples are dissected from other tissues and minced into pieces of about 10 mg in weight. Preadipocytes are isolated by collagenase digestion as described (K.5.1.2). Isolated cell pellets are resuspended in DMEM/Hams F12 medium supplemented with 10% FCS, seeded on membrane inserts ($3.5 \times 10^5/4.3$ cm²) or in a 6-well culture dish, and kept in culture for 16 h. After washing, culture is continued in an adipocyte differentiation medium. After a period of 15 days, 60–80% of seeded preadipocytes develop into differentiated adipose cells as defined by cytoplasm completely filled with small or large LD.

Co-Culture

Co-culture of human fat and muscle cells is conducted according to a recently published protocol (Dietze et al. 2002, Dietze-Schroeder et al. 2005). Briefly, after *in vitro* differentiation of preadipocytes on membrane inserts, the adipocytes are washed once with PBS and then incubated for 24 h in skeletal muscle cell differentiation medium containing 1 pM insulin. Thereafter, adipocytes are washed twice with PBS and individual membrane inserts are subsequently transferred to the culture plates containing differentiated (4 d) myocytes in α -MEM containing 1 pM insulin. This results in

an assembly of the two cell types sharing the culture medium but being separated by the membrane of the insert. The typical distance from the bottom of the culture dish to the membrane is 0.9 mm. Co-culture is conducted for 48 h.

EVALUATION

Integrity of both cell types is routinely checked by light microscopy at the end of the co-culture period. Following co-culture, the effect of insulin or compounds/drugs are studied as described below for normal myocytes and adipocytes.

K.5.1.7

Adipocytes Derived from Mouse Embryonic Fibroblasts (MEF)

It is often useful to study the effects of insulin/compounds/drugs in adipocytes derived from knockout (KO) mice deficient for one (or several) gene(s) relevant for adipocyte differentiation or function. This may lead to the confirmation/exclusion of the affected gene (product) in the molecular mechanism of insulin/compound/drug action on the regulation of lipid metabolism or differentiation in adipocytes. For this, MEF from the KO mice are isolated and differentiated to adipocytes *in vitro*. Stable lines of MEF adipocytes are generated from 12.5–14.5 d embryos of wildtype or homozygous KO mice as described. Selected MEF cells are cultured in DMEM with 10% FBS. At confluence cells are exposed to a pro-differentiation regimen 1 μ M dexamethasone, 5 μ g/ml insulin, 0.5 mM IBMX and 5 μ M rosiglitazone. After two days cells are maintained in medium containing insulin for 4 d, then in medium without insulin for an additional 3–4 d before use.

K.5.1.8

Brown Adipocytes

Brown fat precursor cells are isolated from the interscapular brown adipose tissue of 5–6 week old C57BL/6 wildtype, KO or transgenic mice. Cells are cultured in maintenance medium (DMEM, 10% FBS, 10 mM HEPES, 100 nM sodium selenite, 3 nM insulin, 15 mM ascorbic acid, 50 μ g/ml tetracycline, 50 μ g/ml streptomycin, 50 μ g/ml ampicillin 1 μ g/ml Fungizone [Invitrogen]). Precursors are seeded at 15–20,000 cells/cm² in 12-well plates or in 35-mm coverslip-bottom dishes for biochemical and cell biological assays. After they attained confluence (d 0), cells are cultured for two days in differentiation medium (DMEM,

0.5 mM IBMX, 0.5 mM dexamethasone, 125 μ M indomethacin after which they are maintained in maintenance medium for an additional three days before use.

K.5.1.9

Conditionally Immortalized Cell Strains

PURPOSE AND RATIONALE

Cell lines provide the cell count and homogenous populations for throughput applications, but do not behave as a primary cell can. It is well accepted that primary cells behave more normally than cell lines. Yet, key limitations exist that preclude one from using primary cells during initial phases of drug discovery. The limitations include cost, homogeneity of cell population and total cell count achievable, donor-to-donor variability, and loss of key cellular behaviours when the cell is outside its home organ.

Conditionally immortalized cell strains are primary cells that behave normally in cell culture, can be expanded beyond 40 population doublings, yet maintain normal behaviours throughout the culture time. This is achieved by combining the telomerase rejuvenating effect of telomerase with a specifically modified temperature-sensitive large-T (ts LT) antigen in the same cell. The ts LT mutant codes for a gene product that is functional at 33°C but non-functional at 37°C. Though the construct overcomes the cells' natural senescence behaviour at low passage and provides a strong proliferative signal, the cells are not able to exhibit a full range of differentiated functions. By switching the culture conditions to 37°C one stops cell proliferation and the cells are responsive to media growth factors that drive differentiation. If the line is carried at 37°C for sufficient time, the strain permanently loses its ability to proliferate. A second mutation in the construct controls SV40's ability to auto-excise. Consequently, this mutant conveys a higher level of karyotype stability to the host genomic background over multiple population doublings. Telomerase, also called telomere terminal transferase, elongates chromosomes by adding TTAGGG sequences to the end of existing chromosomes thereby preventing chromosomes from losing base pair sequences at their ends and from fusing to each other. However, each time a cell divides, some of the telomere is lost (25–200 bp per division). When the telomere becomes too short, the chromosome reaches a critical length and can no longer replicate. At this point cells senescence and may be removed by apoptosis. In somatic and aging cells telomerase activity is usually very low. If telomerase is activated in cell, the cell will continue to grow and divide. Consequently,

the conditionally immortalized cell strain with active telomerase acts like a primary cell but provides homogenous cell populations at the high cell counts of cell lines.

EXPERIMENTAL PROCEDURES

Sequential retroviral (MMLV-based vector possessing the Neomycin selection marker) transfection at low passage using ts LT (u197sA58), followed by the telomerase construct hTERT, leads to highly proliferative strains which are highly responsive to differentiation. The conditional immortalization allows at a permissive temperature production of large uniform cell populations while active telomerase is reversing telomere shortening. Concurrently, at the permissive temperature ts LT is actively driving cell proliferation while maintaining karyotype stability over >40 population doublings. By increasing the culture temperature the cells stop dividing. One then converts to a differentiation medium so that the cells can express normal differentiated function and phenotype once again.

Conditionally Immortalized Human Skeletal Muscle Cells

The XM13A1 cell line introduced from Cambrex, Clonetics Bio Science Walkersville Inc. contains a mixed cell population derived from human skeletal muscle of normal 30–40 year old female subjects. These cells can finally be differentiated from myoblasts into well-developed myotubes (e. g. in 96-well plates) that have morphological and phenotypic characteristics of differentiated muscle. These include creatine kinase activity and stimulation (2- to 2.5-fold above basal) of glycogen synthesis by 0.1–1 μ M insulin up to passage 21. Other markers of differentiation found include myogenin, GLUT4, uncoupling protein-3 and GS. Functional activities include stimulation of glycogen synthesis by LiCl and IGF-1, stimulation of glucose transport by AICAR and induction of insulin resistance by glucosamine (see K.5.1.3) or fatty acids.

Conditionally Immortalized Human Preadipocytes

The XA15A1 and XM18B1 cell lines introduced from Cambrex, Clonetics Bio Science Walkersville Inc. represent mixed cell populations derived from subcutaneous adipose tissue of normal 31–40/60 year old male subjects. Both strains are highly responsive, given the correct media conditions (even in the absence of PPAR γ ligands glitazones), to differentiate from the primary normal human preadipocytes into functional mature adipocytes after more than 40 population doublings in culture. Markers of differentiation found include glycerol-3-phosphate dehydrogenase and aP2.

The differentiated cells secrete leptin and adiponectin into the culture medium and exhibit insulin stimulation of glycogen synthesis (up to 2-fold with EC₅₀ of 10–50 nM) and of glucose transport (up to 2.5-fold at 1 μ M).

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K.5.2

Liver and Hepatocytes

GENERAL CONSIDERATIONS

In type II diabetic patients with moderate fasting hyperglycemia, the liver production of glucose is increased by about 50 g/d above normal (DeFronzo et al. 1992). This modest increase is the consequence of reduced suppression of hepatic glucose production by insulin (Firth et al. 1987) and could be nullified by reducing dietary carbohydrate by 50 g/d. The increased hepatic glucose production in type II diabetes is probably caused by a combination of lack of an insulin-mediated reduction in glucagon secretion (Müller et al. 1970) and hepatic resistance to insulin action at the level of both the insulin signaling transduction cascade and its coupling to the metabolic end effector enzymes of gluconeogenesis. The importance of increased hepatic glucose production is underlined by the fact that when phosphoenolpyruvate carboxykinase (PEPCK), the key rate-limiting regulatory enzyme of gluconeogenesis, is overexpressed in mice carrying a transgene expressing the protein hyperglycemia results (Valera et al. 1994). Independent of whether increased hepatic glucose production plays a primary or only secondary role in the pathogenesis of human type II diabetes, analysis of insulin signaling and metabolic action in liver is important for gaining a complete picture of the pathophysiology of type II diabetes. The model of the isolated perfused rat liver offers advantages in determining insulin action, which, compared to primary and cultured liver cells, are the intact tissue, the near physiological function of the liver, and, compared to the *in vivo* situation, the separation from other effects which may also affect hepatic metabolism, like basal glucagon secretion from α -cells, increased sympathetic activity and/or hypothalamic effects (Nonogaki 2000). Taken together, liver cells harbor a number of important targets for compounds/drugs with insulin-like and anti-diabetic activity, which can be assayed using the intact liver and primary or cultured hepatocytes of human and rodent origin.

K.5.2.1

Perfused Rat Liver

Male Wistar rats weighing 200–250 g are anesthetized with 150 mg/kg hexobarbital intraperitoneally. After opening the abdomen, two ligatures are tied around

the stomach, one around the esophageal end to include the adjacent blood vessels and the other around the pylorus. The stomach is removed between these ligatures, a procedure that facilitates the remaining dissection and the subsequent removal of the liver from the animal. A ligature is placed around the bile duct. The portal vein is tied and cannulated with PP10 tubing. The thorax is opened and the vena cava is cannulated via the right atrium. The lower vena inferior cava is tied and the liver is removed from the animal. From the portal vein the liver is washed with 100 ml heparinized (5 IU/ml) physiological saline solution at 37°C for 3 min whilst the outflow occurs via the vena cava. The preparation is then transferred to a perfusion apparatus, where the portal vein cannula is attached to tubing containing the oxygenated medium.

KRBH with 25% bovine erythrocytes, 1.6% BSA, and 22.5 mM Na-L-lactate is used as perfusion medium. The perfusion rate is 30 ml/min. 70 ml of this solution are used for recirculation over 2 h. The test compounds/drug candidates are added in a concentration of 40–100 µM to the perfusate medium. The central element of the perfusion apparatus is a gas-tight, thermostated, double-walled suction filter with an insertable sieve base as support of the organ. The discharge tube is elongated with a Plexiglas tube of 18 cm length and 10 mm inside diameter. The lower end of this tube is connected to a peristaltic pump by means of a Luer safety joint. The suction filter for perfusion of the liver has an internal diameter of 95 mm. On the return of the perfusate to the organ, it passes through a heat exchanger (glass spiral) and a filter holder with a sieve membrane of stainless steel (diameter 25 mm, mesh size 50 µm). A variable carbogen/oxygen mixture, the ratio of which is depending on the pH value of the perfusate, is used for gassing. The perfusate in the Plexiglas tube is bubbled with 70 ml gas mixture/min. To avoid foam formation, a detergent (14 µl/ml 0.1% Genapol PF-10) has to be added to the perfusate. Samples for analyses are withdrawn by catheter immediately in front of the Luer joint in the Plexiglas tube.

K.5.2.2

Primary Rat Hepatocytes

Sprague-Dawley rats (100–200 g) are fed *ad libitum* prior to the hepatocyte isolation. Hepatocytes are isolated from rat livers thoroughly perfused with buffer containing collagenase, hyaluronidase and trypsin inhibitor as described by Van den Berghe and coworkers (1980). The rat livers are perfused for 4 min at 25 ml/min flow rate with perfusion buffer and then

with collagenase buffer until the digestion is complete (~6 min). At the end of the perfusion, connective tissue and large blood vessels are removed. The hepatocytes are then passed through a 100-µm nylon mesh sieve. The cells are washed twice with 125 ml of wash buffer and collected by centrifugation (50 × g, 2 min). The hepatocytes are suspended in plating medium (DMEM, 10% FBS, 100 nM insulin, 25 nM dexamethasone, 6.3 µg/ml transferrin, 22 µg/l gentamycin) and passed through a 100-µm nylon mesh sieve. Cells (1.6×10^5) are plated in 48-well cell culture plates for the various metabolic assays.

K.5.2.3

Cultured Human Hepatocytes

HepG2/C3A (ATCC) human hepatoma cells are cultured in minimal essential medium (α -MEM, GIBCO supplemented with 10% FBS), 100 µM non-essential amino acid mix, 1 mM sodium pyruvate, and 1 mM L-glutamine and are seeded in 48-well plates at 1.4×10^5 cells per well three days prior to the experiment.

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K.5.3

Muscle Tissue and Myocytes

GENERAL CONSIDERATIONS

Muscle cell lines have been used to study skeletal muscle glucose metabolism in response to acute insulin stimulation. Subcellular fractionation followed by immunoblotting as well as photoaffinity labeling followed by immunoprecipitation has demonstrated that in cultured muscle cells insulin causes rapid translocation of the glucose transporter isoform, GLUT4, to the plasma membrane as has been well established for primary and cultured adipocytes. Over decades,

numerous studies have demonstrated that insulin affects many signalling events (e. g. IR and insulin receptor substrate tyrosine phosphorylation) as well as metabolic end effector systems (e. g. glucose transport and glycogen synthesis) in similar fashion. Despite the considerably higher complexity in gaining access to insulin-responsive cells/tissues from muscle compared to adipose tissue, the eminent physiological role of the human skeletal muscle as the predominant acute and short-term storage site for the daily carbohydrate intake necessitates the use of adequate cell- and tissue-based muscle model systems for assaying the insulin-like activity of compounds/candidate drugs. Moreover, these systems may be helpful for analysis of the molecular mechanisms of muscle insulin resistance and of its abrogation by putative insulin-sensitizing compounds/drugs. According to recent findings culminating in the “lipotoxicity hypothesis” (see K.6.3.4), peripheral insulin resistance seems to be correlated to the deposition or loss, respectively, of TAG or its precursors/degradation products in the cytoplasm of skeletal muscle cells, the so-called intramyocellular lipids. Thus, cell- and tissue-based muscle assay systems are also prerequisite for the analysis of myocellular lipid metabolism.

K.5.3.1

Perfused Rat Hindlimb

Female Wistar rats weighing 170–230 g are starved 48 h before the experiment. They are anesthetized by i.p. injection of 50 mg/kg pentobarbital. After a midline incision, the skin is reflected and the superficial epigastric vessels are ligated. The abdominal wall is then incised from the pubic symphysis to the xyphoid process using electrocautery. After ligation of the uterine, ovarian and inferior mesenteric arteries, the upper half of the uterus, the ovaries and part of the descending colon are excised, together with adhering adipose tissue. Next, branches of the hypogastric and pudogastric trunks supplying pelvic viscera are ligated. Ligatures are also placed around the neck of the bladder and the residual portions of the uterus and descending colon. Adipose tissue in the perineal and retroperitoneal regions is removed.

Two pairs of ligatures are placed around the aorta and the vena cava, one just above the origin of the ilio-lumbar vessels and the other above the origin of the renal vessels. The inferior epigastric, ilio-lumbar and renal vessels are then ligated as are the coeliac axis and the portal vein. A ligature is also placed around the tail. The ligatures previously placed around the vena

cava and aorta above the origin of the renal vessels are then tied. The aorta is incised between the left renal and ilio-lumbar vessels and a no. 18 polyethylene catheter filled with 0.85% NaCl containing 200 units of heparin/ml is introduced, passed to a point midway between the ilio-lumbar vessels and the aortic bifurcation, and after flushing with heparin-NaCl solution finally tied in place. The vena cava is cannulated with a no. 16 needle which is secured in a position so that its tip is at the same level as the aortic catheter. The needle is connected with a transparent vinyl tubing. The preparation is then transferred to a perfusion apparatus, where the aorta cannula is attached to tubing containing the oxygenated medium.

KRBH with 25% bovine erythrocytes, 4% BSA, and 10 mM D-glucose is used as perfusion medium. The perfusion rate is 8 ml/min. 70 ml are used for recirculation over 2 h. The test compounds/drug candidate are added in a concentration of 40–100 μ M to the perfusate medium. The central element of the perfusion apparatus is a gas-tight, thermostated, double-walled suction filter with an insertable sieve base as support of the organ. The discharge tube is elongated with a Plexiglas tube of 18 cm length and 10 mm inside diameter. The lower end of this tube is connected to a peristaltic pump by means of a Luer safety joint. The suction filter for perfusion of the hind limb has an internal diameter of 145 mm. On the return of the perfusate to the organ, it passes through a heat exchanger (glass spiral) and a filter holder with a sieve membrane of stainless steel (diameter 25 mm, mesh size 50 μ m). A variable carbogen/oxygen mixture, the ratio of which is depending on the pH value of the perfusate, is used for gassing. The perfusate in the Plexiglas tube is bubbled with 70 ml gas mixture/min. To avoid foam formation, a detergent (14 μ l/ml 0.1% Genapol PF-10) has to be added to the perfusate. Samples for analyses are withdrawn by catheter immediately in front of the Luer joint in the Plexiglas tube.

K.5.3.2

Rat Diaphragms

Sprague Dawley rats weighing 70–100 g are used. The animals are sacrificed during anesthesia and the diaphragms still attached to the rib cages are carefully removed, released from the rib cages and adhering connective and fat tissues, washed in PBS, spread out and divided into two equal pieces as described by Müller and coworkers (1994). For assaying the effects of insulin/compounds/drugs, the hemidiaphragms are incubated in KRH buffer gassed with

carbogen (95% O₂/5% CO₂) in the presence of 5 mM glucose or other ingredients as indicated for the specific assays. In some cases, diaphragms attached to the rib cage instead of hemidiaphragms are used to prevent spontaneous contraction to less than physiological length.

K.5.3.3

Rat Soleus and Extensor Digitorum Longus

In studies of soleus muscle and extensor digitorum longus (EDL), the hindquarters are perfused for 3 min (25 ml/min) to wash out the blood. The two soleus or EDL muscles from each rat are removed with tendons intact, placed in perforated baskets and incubated in separate test tubes. Muscles from three rats are incubated together with insulin/compounds/drug candidates. The perfusing and incubation medium is KRB (pH 7.4) containing 8 mM glucose, 1 mM pyruvate and 0.2% (w/v) BSA. The incubation medium was gassed continuously with 95% O₂/5% CO₂. At the end of the incubation, muscles are freeze-clamped with aluminium tongs cooled in liquid nitrogen, and then trimmed of connective tissue and visible fat while kept in liquid nitrogen. In order to further ensure that findings reflected the biology of muscle cells, these are before analysis in most experiments isolated from other tissue components by microdissection using a stereomicroscope after freeze-drying.

K.5.3.4

Human Muscle Strips

A human muscle preparation has been introduced successfully by Dohm and coworkers (1988). For this, immediately after the surgical incision, muscle strips (abdominal rectus muscle) are mounted at *in vivo* length by using one set of clamps, which is 2.5-cm wide and constructed from two pairs of hemostats. The clamp is placed by the surgeon on the muscle, and a 0.5–1 g muscle piece is clamped, excised, and immediately transferred to oxygenated (KRBH) for immediate transfer to the laboratory. Muscle fiber strips, weighing between 20 and 50 mg, are dissected free from the mounted specimen, secured with a small Plexiglas support, and cut free. Particular care has to be taken to remove all visible fat. From one muscle piece, it is possible to obtain 5–10 muscle strips. After preparation, the muscle strips are washed for 30 min in KRBH supplemented with 38 mM mannitol, 2 mM pyruvate, and 10 mg/l BSA. The viability of muscle is usually investigated by analysis of

3–O-methylglucose transport (see below). Typically, insulin-stimulated glucose transport follows concentration-dependent fashion with the maximum effect of about 2-fold above the basal glucose transport rate ($\sim 1 \mu\text{mol/ml} \cdot \text{h}$).

K.5.3.5

Cultured Human Skeletal Muscle Cells

Human skeletal muscle cell cultures are handled as described by Aas and coworkers (2004). A cell bank of satellite cells is established from muscle biopsy samples of the vastus lateralis muscle from four healthy volunteers (age 25–30 years, BMI 22–25, fasting glucose and insulin within the normal range and no family history of diabetes). The biopsies have to be obtained with informed consent and approval by the National Committee for Research Ethics. Muscle cell cultures free of fibroblasts were established as previously described (Henry et al. 1995) with minor modifications. Briefly, muscle tissue is dissected in Ham's F-10 media at 4°C, dissociated by three successive treatments with 0.05% trypsin/EDTA, and then satellite cells were resuspended in skeletal cell growth medium with 2% FCS and no added insulin. The cells are grown on culture wells coated with extracellular matrix gel (Gaster et al. 2001). After 2 to 3 weeks at about 80% confluence, fusion of myoblasts into multinucleated myotubes is achieved by growth for 8 d in α -MEM with 2% FCS. Hyperglycaemic medium can be made by the addition of glucose (to a concentration of 10 or 20 mM) to α -MEM with 2% FCS. All cells used in the experiments should be at passage 3 to 6.

K.5.3.6

L6 Myotubes

Stock cultures of L6 rat skeletal muscle cells from the ATCC are grown in α -MEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and antimycotics (growth medium) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. For the experiments, stocks are trypsinized and the myocytes reseeded on 6-well cell culture plates or 60 \times 15 mm Petri dishes at a density of 4,000 cells/cm². After 24 h ($\sim 80\%$ confluence), the medium is changed to α -MEM containing 2% (v/v) FBS and antibiotics/antimycotics as described above (differentiation medium) that is replaced after 2, 4, and 6 d of culture. After 7 d, myotube differentiation is complete, and experimental procedures are initiated. In all experiments, L6 myotubes are serum-starved for 4 h before expo-

sure to insulin/compounds/drugs. All controls are incubated with equal concentrations of the vehicle (e. g. DMSO) and the respective concentrations of fat-free albumin (e. g. BSA) as present in the treated cells.

K.5.3.7

L6 Myotubes Transfected with GLUT4

L6 myotubes express a detectable but rather moderate amount of the insulin-regulated GLUT4 which may account, in part, for the limited insulin stimulation of their glucose transport system. To increase the insulin responsiveness of glucose transport, L6 myocytes are transfected with myc-tagged GLUT4 (L6 GLUT4-myc cells) (Dr. Amira Klip, Davison of Cell Biology, The Hospital for Sick Children, Toronto, Canada). After growth in 96-well tissue culture plates using 200 μ l of α -MEM containing 2% FBS, 100 units/ml penicillin G, and 100 mg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 8.5% CO₂, myoblasts are seeded in medium containing 2% (v/v) FBS at a density of $\sim 2 \times 10^4$ cells/ml and used 6–8 d postseeding. Cells are fed fresh medium every 48 h and used at the stage of myocytes or after the differentiation to myotubes.

K.5.3.8

BC₃H₁ Myocytes

GENERAL CONSIDERATIONS

The BC₃H₁ cell line is a non-fusing spontaneously and reversibly differentiating mouse muscle cell line derived from a mouse neoplasm (Schubert et al. 1974). As for other cultured cell lines, the BC₃H₁ myocytes demonstrate intermediate characteristics, possessing electron-microscopic features of both smooth and skeletal muscle but with a nicotinic acetylcholine receptor and an action potential more characteristic of skeletal muscle (Standaert et al. 1984). Unfortunately, BC₃H₁ myocytes do not express GLUT4 and therefore the limited insulin stimulation of glucose transport relies on the glucose transporter isoform 1, GLUT1, exclusively.

PROCEDURE

BC₃H₁ myocytes are cultured to confluence in 100-mm dishes over 10–14 d in DMEM supplemented with 15% "Process Serum Replacement-I" (Sigma) and 25 mM glucose are added 18 h before the experiment. Cells are rinsed and pre-incubated at 37°C for 20 min in Dulbecco's PBS with 0.1 mM CaCl₂ and 1 mg/ml

BSA, then treated with vehicle or compounds/drug candidates in vehicle for 30 min or overnight.

K.5.3.9

C₂C₁₂ Myotubes

PURPOSE AND RATIONALE

C₂C₁₂ cells, a mouse skeletal muscle cell line, has been isolated from dystrophic mouse muscle by Yaffe and Saxel (1977), McMahon and coworkers (1994), Ernst and White (1996). Subsequently, the myocytes have been shown to express a limited amount of GLUT4 and to be suitable for stable transfection experiments of exogenous cDNA, making this cell line a candidate for stable transfections of cDNAs that encode mutant skeletal muscle and cardiac protein isoforms.

PROCEDURE

Monolayers of mouse C₂C₁₂ myoblasts (ATCC CRL-1772) are grown in Dulbecco's modified high-glucose Eagle's medium (DMEMH; 90%/10% (v/v) FBS/4.0 mM glutamine/50 μ g/ml gentamycin, in a humidified incubator at 37°C, 5% CO₂ as has been described by Muoio and coworkers (1999) Cells are grown in 100-mm dishes, sub-cultured at 60–80% confluence, and split at a ratio of 1:10 using trypsin (0.25% (w/v) in MEM with 1.0 mM EDTA). Cells grown to 60% confluence are sub-cultured at a ratio of 1:15 into 6-well dishes that had been coated with 0.01% (w/v) collagen. When cells are 80% confluent, myoblasts are induced to differentiate into myotubes by changing to low-serum differentiation medium (98% DMEMH/2% (v/v) horse serum/4.0 mM glutamine/25 mM HEPES/50 μ g/ml gentamycin). Differentiation medium is changed daily. By day 5, cells are typically fully confluent and had differentiated into multinucleated, contracting myotubes.

K.5.3.10

Cardiomyocytes

Cardiomyocytes are isolated by perfusion of hearts from adult rats with collagenase according to established protocols (Eckel et al. 1983 and 1991, Bähr et al. 1995).

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K.5.4

Pancreas and Pancreatic β -Cells

GENERAL CONSIDERATIONS

Concurrent with the obesity epidemic, the incidence of type II diabetes is increasing at an alarming rate. Type II diabetes arises when the endocrine pancreas fails to secrete sufficient insulin to cope with the metabolic demand, because of acquired β -cell secretory dysfunction and/or decreased β -cell mass. Insulin secretory dysfunction is well documented. Whether insulin secretory dysfunction is a cause or consequence of the disease (in response to chronic challenge for compensation of the peripheral insulin resistance) is still debated, but there is mounting evidence that it may be

symptomatic of changes in β -cell mass. Although proposed 50 years ago, the hypothesis that β -cell loss plays an important role in the pathogenesis of type II diabetes has only recently come to the fore. β -cell mass in the adult is plastic, and adjustments in β -cell growth and survival maintain a balance between insulin supply and metabolic demand. For example, obese individuals who do not develop diabetes exhibit an increase in β -cell mass that appears to compensate for the increased metabolic load and obesity-associated insulin resistance. However, this β -cell adaptation eventually fails in the subset of obese individuals who develop type II diabetes. Indeed, most individuals with type II diabetes whether obese or lean, show a net decrease in β -cell mass. Thus, type II diabetes is a disease of relative insulin deficiency. Given the pivotal role of β -cell mass in determining whether an individual will progress to type II diabetes, there is growing interest in understanding the mechanism that controls the life and death of β -cells. The molecular mechanisms involved in the decision between β -cell life and death have been reviewed by Rhodes (2005). Furthermore, assay systems based on the isolated perfused pancreas, perfused islets, insulinoma cells and cultured β -cells are required to study the effects of compounds/drugs on insulin secretion, β -cell growth and β -cell survival.

K.5.4.1

Perfused Rat Pancreas

PROCEDURE

Male Wistar rats (e. g. Hoe:WISKf, SPF 71-strain) weighing 200–250 g serve as donors. Prior to the experiment, the animals have free access to food and water. The pancreas is removed under pentobarbital (50 mg/kg *i.p.*) anesthesia. The mesenteric artery is doubly ligated and cut, and the entire intestine below the duodenum is separated and removed from the rat to simplify the exposure. The esophagus is ligated as high as possible and cut above the ligature. A loose ligature is placed, but not tied, around the entire gastrohepatic ligament. The aorta is cautiously exposed through the crura of the diaphragm well above the point of origin of the celiac axis. The ligature around the gastrohepatic ligament is then tied as tightly as possible, and the ligament cut above the ligature. The aorta is cut between the double clamps and below the lower clamp, and the preparation is lifted out from the abdomen. The clamps are removed from the aorta which is slit open on the side opposite to the origin of the celiac axis, revealing the opening of the latter. The arterial cannula

is inserted and tied in place. An opening is made close to the ligature, as near as the end of the portal vein as possible.

EVALUATION

Circulation through the preparation is initiated and fluid is observed to flow from the slit in the portal vein. After a minute or two, the flow is stopped and a cannula is inserted into the portal vein and tied in place. Flow is then resumed at a perfusion pressure of about 100 mm Hg. Carbogenated Krebs-Ringer bicarbonate buffer with 2% BSA and 5.5 mM glucose is used as perfusion medium at a temperature of 37.5°C with a perfusion rate of 1.75 ml/min. The perfusate is collected every minute for 30 min. After 5 min perfusion with 5.5 mM glucose, the test compound is added until the 15th min (concentrations between 0.05 and 0.5 mM for highly active substances), followed by perfusion with 5.5 mM glucose and finally with 16.6 mM glucose. The perfusate medium samples are stored at -20°C until further processing.

K.5.4.2

Perifused Islets

Male Wistar rats weighing 200–250 g serve as donors. Before the study, the animals have free access to food and water. Two donor rats are used for each test. The pancreas is removed under pentobarbital anesthesia. The islets are obtained by the collagenase (e.g. collagenase type IV, Worthington) method and collected under a stereomicroscope. In each test, up to 10 chambers each with 15 islets are perifused. Cut-off Microfuge tubes, sealed with Tuohy-Borst adapters, serve as perfusion chambers. Two thick-walled, small-diameter Teflon catheters are passed through the adapter into the chamber. One of the catheters extends to the bottom of the chamber and acts as the perfusate inlet, the other extends to the lower edge of the adapter cone and acts as outlet. The latter is connected to a multi-channel peristaltic pump which delivers the perfusate to a fraction collector. The chamber volume is 0.15 ml. The perfusate flow rate is 0.1 ml/min. The perfusate consists of a carbogenated Krebs-Ringer bicarbonate buffer with 1.0 mM glucose, 0.25% BSA, and 5 mM theophylline. The storage vessels for the perfusate, the chambers, and the inlet catheters are immersed in a water bath of 37°C.

EVALUATION

After a pre-perfusion phase of one hour, the perfusate is collected every min for 46 min. From the 2nd until

the 18th min, the test compound is added at concentrations between 0.1 and 2.5 μ M, and from the 34th to the 46th min, the glucose concentration is raised to 20.0 mM. The hormones: insulin, glucagon and somatostatin are determined by using a commercially available RIA-kit or ELISA-kit.

K.5.4.3

Insulinoma Cells

PURPOSE AND RATIONALE

Chick and coworkers (1977) described a transplantable insulinoma in the rat which was originally observed as primary tumor in the pancreas of an old male NEDH albino rat being previously irradiated during a parabiosis experiment. Fragments of this tumor were transplanted to young NEDH rats in many passages inducing severe hypoglycemia in host rats. Gazdar and coworkers (1980) reported the establishment of a continuous cell line of a rat islet cell tumor which secretes primarily insulin and some somatostatin. Bhatena and coworkers (1982) studied insulin, glucagon, and somatostatin receptors on cultured cells and clones from rat islet cell tumor.

PROCEDURE

The insulinoma is transplanted when the tumor has reached a diameter of about 2 cm and the carrier animal exhibits distinct manifestations of insulin excess. The tumor is removed, cut open, mixed briefly in a mortar and one tenth to one twentieth is injected between the shoulder blades of another animal. No metastases are observed. The histological examination also shows a nonmalignant adenoma. The tumors grow only in rats of the strain NEDH (New England Deaconess Hospital).

K.5.4.4

Cultured β -Cells

GENERAL CONSIDERATIONS

Reliable β -cell models are of paramount importance for diabetes research (see above). It is generally accepted that the use of primary cells is preferable. However, this requires large quantities of isolated pancreatic islets, which is work-intensive and has the inherent inconvenience of representing a mixed population of β -, α -, δ -, and F cells. Consequently, rodent β -cell lines have proven their usefulness, and their continuous development is still essential until clonal human β -cells become available. The intrinsic challenge when establishing a β -cell line is the maintenance of

tissue-specific differentiation combined with adequate cell proliferation. As a result, only a limited number of β -cell lines are available to date, all of rodent origin. Several insulin-secreting cell lines are reported in the literature (review by Poitout et al. 1996). The most widely used are RINm5F, β H9C9, β TC6-F7 and, more recently, MIN6. Among the mouse-derived β -cell lines (Miyazaki et al. 1990, Knaack et al. 1994), MIN6 cells (Miyazaki et al. 1990, Ishihara et al. 1993) represent a valuable model, which was further improved by isolation of the clonal subline MIN6m9 (Minami et al. 2000). However, MIN6 cells exhibit secretory responses to pyruvate, which is not a secretagogue for normal islets (Skelly et al. 1998). Mouse β H9C9 grow very slowly and are thus difficult to study. Of rat origin, RINm5F cells are poorly differentiated, have low insulin content and do not respond to glucose in the physiological concentration range (Halban et al. 1983, Praz et al. 1983, McClenaghan et al. 1998), and BRIN-BD11 cells are poorly differentiated, exhibiting low insulin content and weak secretory responses to glucose (McClenaghan and Flatt, 1999). Finally, loss of differentiated features as a function of time in tissue culture has been reported for several rodent cell lines, including RIN1046–38 and β TC6 (Clark et al. 1990, Ferber et al. 1994). Genetic engineering of RIN1046–38 cells results in clones with stable glucose responsiveness but with maximal insulin secretion occurring at subphysiological glucose concentrations because of a high level of low K_m hexokinase activity in these cells (Ferber et al. 1994, Hohmeier et al. 1997). Stable glucose responsiveness has also been reported for β TC cells after clonal selection in soft agar (Knaack et al. 1994), but even these cloned cell lines (e. g. β TC6-F7) appear to lose glucose responsiveness after prolonged tissue culture (Zhou et al. 1998). Currently, clonal INS-1 cells (e. g. INS1–832/13 or 1E; Hohmeier et al. 2000) appear to represent the most attractive vehicles for studying β -cell function.

PROCEDURE

RINm5F

Insulin-producing cells from the RINm5F cell line are grown in RPMI medium containing 10% (v/v) heat inactivated FCS, 50 IU/ml penicillin, 0.25 μ g/ml amphotericin B, and 50 μ g/ml streptomycin at 37°C in an atmosphere of humidified air/CO₂ (19:1) (Praz et al. 1983). The cells are seeded at a density of 3.5×10^4 cells/ml in 20 ml of medium (75 cm² culture flasks). The medium is replaced 4 times a week (one passage). Thereafter, the cells are treated with trypsin (0.02% trypsin in 0.9% NaCl/0.2 mM EDTA)

for 2–5 min at 37°C. The trypsin-treated cells are diluted, re-seeded at a density of 2×10^6 cells per 75 cm² culture flask and grown to 70% confluency.

For single-cell patch clamp studies, RINm5F cells are maintained in RPMI 1640 tissue culture media, containing 11 mM glucose, supplemented with 10% FCS, 2 mM glutamine and 50 μ g/ml gentamycin. Cells are seeded out every 2–3 d onto Petri dishes and kept in a humidified atmosphere of 95% O₂ and 5% CO₂ at a temperature of 37°C. The cells are isolated by incubation in a Ca²⁺-free medium containing 0.25% trypsin for about 3 min. Single cells and clusters of 2–3 cells are obtained after centrifugation with 800 rpm and are stored on ice until use. The tight-seal whole-cell patch-clamp technique can be applied to single cells.

MIN6

The MIN6 cell has been derived from transgenic mice expressing a comparable transgene (Miyazaki et al. 1990). MIN6 cells cultured on a gelatine layer have been shown to form spherical cell clusters (pseudo-islets) similar to those in islets. (Hauge-Evans et al. 1999). Two rodent insulin genes have been identified in MIN6 cell pseudo-islets (Roderigo-Milne et al. 2002). The glucose-induced biphasic insulin secretion pattern observed in isolated mouse islets was also demonstrated in MIN6 pseudo-islets. Differences between pancreatic islets representing a complete endocrine organ and the artificial MIN6-pseudo-islets containing only β -cells were observed cautioning the use of the pseudo-islets for evaluation of compounds/drugs (Brenner and Mest 2003).

For culture, MIN6 cells (starting passage 40) are maintained in DMEM containing 25 mM glucose and supplemented with 15% heat-inactivated FBS in humidified 5% CO₂/95% air at 37°C. Cells are exposed to glucose-free extracellular solution for 30 min prior to measurement of insulin secretion or intracellular calcium.

Palmitate-Treated MIN6

Busch and coworkers (2005) studied the effect of fatty acids on the viability and functionality of pancreatic β -cells. For this, palmitate-treated MIN6 cell pools are selected and routinely passaged in 75-cm² flasks with 20 ml DMEM containing 25 mM glucose, 24 mM NaHCO₃, 10 mM HEPES, 10% (by vol.) FCS, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 1 mM palmitate coupled to 2.3% BSA (wt/vol). This selection procedure is carried out twice, giving rise to two independent pools of palmitate-treated cells. Both pools

are used for transcript profiling experiments and functional studies. Control MIN6 cells are taken through the same selection process but using BSA-medium without palmitate. Cells are seeded in DMEM as follows. For [^{14}C]palmitate-labeling and protein measurement, 2×10^5 cells per well in 24-well dishes (0.5 ml) are used, for microarray experiments, 5×10^6 cells per 25-cm² flask (5 ml) are used, for analysis of lipid composition, 6×10^6 cell per 10-cm dish (15 ml) are used. At 48 h before the experiments (24 h after seeding), the medium is replaced with DMEM (as above but with 5 mM glucose) supplemented with either BSA alone or BSA coupled to palmitate. Couplings are prepared in DMEM (25 or 5 mM glucose) as described by Busch and coworkers (2002). The couplings are diluted 1:8 (for culture and for experiments), giving a final concentration of 1 mM palmitate to 2.3% BSA (wt/vol), corresponding to a molar ratio of 3:1.

HIT-T15

The insulin secreting HIT cell line has been developed by Santerre and coworkers (1981) by isolating pancreatic islets from the hamster, dispersing the islets into single cells, transforming the cell isolates with the simian virus 40 (SV40), and cloning out the insulin-secreting cell lines. These clonal cells retain a differentiated function and respond to secretagogues and inhibitors of insulin secretion (Boyd et al. 1991).

INS-1/2

Asfari and coworkers (1992) derived INS-1 cells and INS-2 cells from parental radiation-induced RINm5F cells. In the course of a co-culture of lymphocytes and RINm5F cells in the presence of 2-mercaptoethanol, the authors observed the formation of free-floating cell aggregates which appeared to be morphologically different from the parental cells. These clusters were isolated and gave rise to the INS-cell lines, whose viability is dependent on the presence of 2-mercaptoethanol in the media and whose secretory characteristics are similar to those of native islets with its modulation by free radicals upon challenge of the cells by alloxan has been reported by Janjic and coworkers (1995). Due to the nonclonal nature of these cells, limited stability over passages probably explains some of the discrepancies observed among the numerous laboratories using parental INS-1 cells worldwide. To circumvent this problem, Hohmeier and coworkers (2000) stably transfected INS-1 cells with the human proinsulin gene, followed by selection of clones based on robust glucose-stimulated insulin secretion. The resulting INS-

1-832/13 cells are highly glucose responsive. However, these cells overexpress human insulin driven by the ubiquitous cytomegalovirus promoter in addition to the endogenous rat insulin, rendering it impossible to judge the differentiated state based on insulin content. An alternative approach would be the cloning of well differentiated INS-1 cells without genetic manipulation. Hence, the clonal INS-1E cells has been isolated from the parental cells based on both their insulin content and their secretory response to glucose as well as adequate proliferation (Hohmeier et al. 2002).

INS-1E

Clonal INS-1E cells, derived from parental INS-1 cells (Asfari et al. 1992) on basis of selection for insulin content and adequate proliferation (Janjic et al. 1999), are cultured in a humidified atmosphere containing 5% CO₂ in complete medium composed of RPMI 1640 supplemented with 5% heat-inactivated FCS, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. The maintenance culture is passaged once a week by gentle trypsinization, and cells are seeded at a density of 4×10^4 cells/cm², i.e. 3×10^6 cells, in 75-cm² Falcon bottles with 20 ml complete medium. The potential presence of mycoplasma is regularly checked using a photometric enzyme immunoassay for the detection of PCR-amplified mycoplasma DNA (Roche, Diagnostics). INS-1E are seeded at 2×10^5 cells/ml in Falcon 24-well plates and used 4–5 days thereafter, with one medium change on d 3 or 4. For generation of INS-1E cell clusters, cells are seeded in nonadherent bacterial 10-cm petri dishes and cultured in complete medium for 5–6 d before use. The stable differentiated INS-1E β -cell phenotype was reported for over more than 100 passages corresponding to a 2-year continuous follow-up. INS-1E cells can be safely cultured and used within passages 40–100 with high insulin contents. Glucose-induced insulin secretion is concentration-dependent and similar to rat islet responses as are the secretion responses to amino acids and sulfonylureas. Moreover, INS-1E cells retained the amplifying pathway, as judged by glucose-induced augmentation of insulin release in a depolarized state. Finally, spheroid clusters, sometimes referred to as pseudoislets (Hauge-Evans et al. 1999), composed of reaggregated INS-1E cells, can be prepared and tested for their ability to respond to secretagogues. Thus, INS-1E cells represent a stable and valuable β -cell model for the analysis of insulin secretion induced by compounds/drug/candidates.

β -TC

Hanahan (1985) applied gene transfer technology to the establishment of β -cell lines. In order to target the expression of viral DNA to the β -cell, recombinant oncogenes have been designed by fusion of the 5'-regulatory region of the rat insulin II gene with the early coding region of SV40. The transgenes have been microinjected into fertilized mice embryos, which were then implanted in the oviduct of pseudo-pregnant females. The offspring specifically expressed SV40 in their β -cells and spontaneously developed β -cell tumors at 10–20 weeks of age. Tumors were subsequently excised, isolated and propagated in culture, giving rise to β -tumor cell (β -TC) lines (Erfrat et al. 1988). Because these cells do not grow in culture at low density, they have not been cloned, and several subpopulations have been derived. Hamaguchi and coworkers (1991) applied this technique to the development of the NIT1 cell line from transgenic NOD mice in order to establish an immortalized source of NOD β -cells.

Betacyt

The betacyte, also called the HepG2Ins/ β -cell, is a genetically engineered insulin-secreting human liver cell line that is glucose responsive (Simpson et al. 1995 and 1996, Tuch et al. 1997). The clone was constructed by doubly transfecting the HepG2 cell line with insulin cDNA under the control of the constitutive CMV promoter and the cDNA for the glucose transporter isoform 2 (GLUT2), which is specific to β -cells and hepatocytes. Interestingly, this cell is capable not only of synthesizing, storing and secreting insulin in a regulated fashion when challenged with physiological concentrations of glucose, but also of acting as a liver cell and secreting albumin.

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K.6

Assays for Insulin and Insulin-Like Metabolic Activity

EVALUATION

Data from the metabolic assays (and signaling assays, see below) are calculated as stimulation factor above basal activity (absence of insulin/compound/drug candidate) for processes stimulated (e.g. lipogenesis, glucose transport and GLUT4 translocation) or as difference between the basal and insulin/compound/drug candidate-induced values for processes down-regulated (e.g. lipolysis). In each case, these data, which reflect the responsiveness of the metabolic effector system studied toward the respective stimulus (insulin/compound/drug candidate), are normalized to the basal (set at 0%) and maximal insulin action (set at 100%; elicited by maximally effective concentration of insulin). For characterization of the sensitivity of the metabolic effector system toward the respective stimulus, effective concentrations for the induction of 150% (or higher) of the basal activity (set at 100%) can be given. These so-called EC_{150} -values facilitate the insulin-independent comparison of the relative potency of the insulin-like activity between compounds/drug candidates, in general, and in particular for those frequently observed stimuli, which do not elicit the same maximal response in % stimulation or inhibition and/or fail to approach the maximal insulin response.

Typically, at least three different cell/tissue preparations with two to four independent incubations with insulin/compound/drug candidate and two to four independent activity measurements (i.e. assays) should be performed. Concentration/response curves can be fitted to the equation

$$y = a + b[x/(x + k)]$$

using a Marquardt-Levenberg non-linear least squares algorithm. When plotted on linear-log axes, this equation gives a sigmoidal curve where the parameters are associated with the following properties:

a = basal response;

$a + b$ = maximal response;

k = half-maximal concentration (EC_{50});

x = concentration of insulin.

The term “insulin-like activity” is used in a wide range of definitions varying in the type and number of parameters evaluated by the respective assays which

have to be fulfilled by a given compound/drug candidate. An appropriate criterium for a compound/drug candidate with insulin-like activity may represent that it exerts at least 20% of the maximal insulin response in at least three major metabolic insulin actions (e. g. glucose transport, lipogenesis, anti-lipolysis) in at least one insulin target cell/tissue. However, the final judgement of a compound/drug candidate as being appropriate for further evaluation and development has to rely on the complete data set of its profiling in many metabolic and signaling assays rather than to stick to the fulfillment of fixed criteria for one or a few parameters.

K.6.1

Assays for Insulin and Insulin-Like Activity Based on Adipocytes

K.6.1.1

Differentiation

PURPOSE AND RATIONALE

The increase in obesity and the identification of adipocyte-secreted proteins that regulate energy metabolism (Drevon 2005) have generated huge interest in adipocyte biology. Adipocytes are the primary storage site for energy in vertebrate animals. During fasting, adipocytes release energy-rich molecules that provide metabolic fuels to other tissues. Adipocytes also secrete hormones that orchestrate the storage, release, and oxidation of energy-rich molecules throughout the body and that control behavior, including feeding (Rajala and Scherer 2003). Insulin as anabolic hormone is of critical importance for the differentiation of adipocytes in humans. Consequently, assays that monitor the quantitative analysis of adipocyte differentiation may be helpful for studying the effects of insulin/compounds/drugs on pathways which are engaged both directly in the regulation of the differentiation and in the molecular mechanisms of metabolic insulin action.

PROCEDURE

Understanding the molecular mechanism underlying adipocyte differentiation has been one of the major focuses of many researchers in the field of metabolic disorders. Methods in use for the assessment of fat cell development and maturation after initiation of adipogenesis in cell culture include microscopic examination of cellular LD formation and cellular lipid staining by Oil Red O (von Goor et al. 1986). These methods, although excellent in detecting the presence of

intracellular lipid, are ineffective in objectively quantifying the degree of fat accumulation if not used in conjunction with other extraction and analytic systems. Particularly, during the process of adipogenesis of preadipocytes, it is observable that cells are heterogeneous in their response to adipogenic agents in terms of speed of adipogenic conversion and degree of fat accumulation (Shigematsu et al. 2001). This heterogeneity of response may cause difficulties when evaluating and comparing the effects of several treatments that target only fat cells of certain status due to the masking influence of those non-targeted cells. Thus, an effective method to detect and classify cells with similar fat content will certainly increase analytical precision in monitoring fat cell development and the ability to quantify the effects of therapeutic agents. Currently, flow cytometry and fluorescence-activated cell sorters (FACSS) are extensively used in the analysis of adipocyte differentiation and lipid accumulation as well as their modulation by compounds/drugs as is exemplified best by the study from Lee and coworkers (2004).

EVALUATION

A simple and sensitive method to detect and quantify lipid accumulation inside cells by flow cytometry has been introduced (Lee et al. 2004). Using this method, elevated levels of cytoplasmic granularity can be detected that correlate well with an increased level of lipid accumulated inside cells after adipogenic conversion. Furthermore, this method is appropriate to monitor and quantify adipose cell maturation within a complex population of cells and to identify and collect the adipose cells with similar lipid storage for further analysis. Flow cytometry offers distinct advantages over existing detection systems for cytoplasmic lipid staining and lipid extraction and can represent a powerful analytical tool to monitor the effect of chemicals and biological molecules, including compounds and putative anti-obesity drugs on adipose cell conversion and maturation. Moreover, in combination with a cell sorting facility, this method offers a simple and efficient means of collecting adipose cells of specific status for further analysis. In conclusion, flow cytometry enables the direct measurement of the lipid content of specific cells in a complex population of adipose cells. Not only is the system simple, sensitive, and quantitative, but it also offers an advantage over the existing methodology, with its capability of monitoring the degree of intracellular lipid accumulation in a precise, fast, and selective manner. These results provide a basis for developing a variety of applications in com-

pound and drug screening procedures to monitor adipose cell development.

K.6.1.2

Lipogenesis

GENERAL CONSIDERATIONS

Lipogenesis encompasses the complete biosynthetic pathways for TAG stored in cytoplasmic LD and phospholipids building up the internal and plasma membranes (including their lipidic intermediary and degradation products). The type as well as the concentration of the labeled precursor (e. g. glucose, fatty acids) whose conversion or incorporation into TAG and phospholipids is followed determines which step(s) of the lipogenic pathway is monitored by the lipogenesis assay (e. g. glucose transport, acylation). Thus, simple variations of the assay conditions allow the selective analysis of individual rate-limiting steps of lipogenesis and their modulation by compounds/drug candidates.

K.6.1.2.1

Method Based on the Incorporation of Radiolabeled Glucose

PURPOSE AND RATIONALE

This assay measures the complete pathway of lipid synthesis (lipogenesis) encompassing the transport of the radiolabelled glucose across the plasma membrane of the adipocytes, its conversion into glycerol and/or fatty acids and their subsequent esterification into predominantly neutral TAG and phospholipids and finally the deposition of TAG in LD in the cytoplasm of the adipocytes. Since in adipocytes each of these steps is stimulated by insulin, albeit to varying degrees, the lipogenesis assay is perfectly suited for the analysis of effects of compounds/drugs on the complex insulin signaling cascade regulating lipogenesis. Dependent on the experimental conditions chosen (i. e. low or high glucose), the assay monitors predominantly the effect of insulin/compound/drug on glucose transport or on the subsequent esterification. At low glucose concentrations (up to 0.1 mM glucose), when according to TLC-analysis of the total TAG after enzymatic digestion the majority of the radiolabel is incorporated into the glycerol backbone, the glucose transport step is rate-limiting and monitored. In contrast, at higher glucose concentrations (above 2 mM), when two thirds of the radiolabel are recovered with the fatty acid moieties of TAG, the rate-limiting and monitored step is the esterification rather than glucose transport, which is driven by mass action. Since in adipocytes

the insulin stimulation of glucose transport is significantly more pronounced than that of esterification, the lipogenesis assay performed at low glucose concentration (<0.1 mM glucose) exhibits the highest insulin responsiveness whereas at intermediary glucose concentrations (0.1–2 mM), it represents a compromise for the measurement of both transport and esterification with intermediary insulin responsiveness and at high glucose concentrations (>2 mM glucose) it predominantly monitors the esterification with lowest insulin responsiveness.

PROCEDURE

For measurement of lipogenesis monitoring effects on both glucose transport and esterification, isolated rat adipocytes are incubated with D- ^3H glucose (0.55 mM final concentration, 0.1–1 μCi). The cells are lysed and the total lipids separated from water-soluble products and the incubation medium including the unincorporated ^3H glucose by addition of toluene-based scintillation cocktail. After phase separation, radioactivity incorporated into total lipids/phospholipids is determined by liquid scintillation counting directly without removal of the lipid phase based on determination of the radiolabel of the lipidic products partitioned into the toluene phase containing the scintillator rather than of the ^3H glucose left in the aqueous phase lacking scintillator (Moody et al. 1974).

The reaction is started by the transfer of 0.2 ml of adipocyte suspension (3.5×10^5 cells/ml) in KRHB to scintillation vials containing 0.1 ml of ^3H glucose (2 $\mu\text{Ci}/\text{ml}$, 4.4 mM), 0.4 ml of 2-fold KRHB and 0.3 ml of insulin or compound/drug with insulin-mimetic activity dissolved in vehicle (e. g. DMSO) and diluted with KRHB to the appropriate concentration of compound and vehicle (e. g. 3% DMSO). The scintillation vials are placed under a stream of carbogen for 10 s, then closed and placed in a very slowly shaking water bath (37°C). After incubation for 90 min, the reaction is terminated by the addition of 10 ml of toluene-based scintillation cocktail. The vials are mixed rigorously using a vortexer and subsequently left standing for 2–4 h to allow phase separation. The ^3H -radioactivity is determined with a liquid scintillation counter.

EVALUATION

Blank values obtained from a typical reaction mixture containing buffer and ^3H glucose but lacking either adipocytes or compound/drug/insulin have to be included in each experiment. Since the quality of the cells decreases with time (resulting in increased lipogenesis in the basal state), it is recommended to

set up 2 basal incubations for every 20–25 test mixtures. In addition, for direct comparisons (e. g. of insulin analogs) incubations with identical (insulin) concentrations should be performed immediately for one agent (insulin) after the other rather than with the complete concentration series for one agent (insulin) after the other. This procedure allows the resolution of potency differences of less than 10% between two insulin analogs as reflected in corresponding shifts of the apparent EC_{50} -values with identical maximal responses. The blank values lacking adipocytes (usually 500–600 dpm) are subtracted from the values measured for the corresponding set of test mixtures containing adipocytes to correct for 3H -radiation originating from the aqueous phase (i. e. [3H]glucose left in the incubation medium). Fold stimulations reflecting the responsiveness of the glucose transport and/or esterification systems of the adipocytes toward insulin/compound/drug are calculated as ratio between the corrected test values (presence of insulin/compound/drug) and basal values (absence of insulin/compound/drug). Typically, insulin induces 15- to 20-fold, 8- to 12-fold and 2.5- to 4-fold stimulations in lipogenesis at 50 μM , 0.55 mM and 2 mM glucose, respectively. Due to the very limited number of pipetting steps (with the most critical one being the transfer of an equal number of cells to each assay mixture), the standard deviations are rather low. Usually, 2–4 measurements per data point are sufficient.

An insulin-concentration/response curve should be performed for each experiment to test the insulin sensitivity and responsiveness (and thus the quality) of the adipocytes prepared. Appropriate insulin concentrations are 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.15, 0.2, 0.5, 1, 5 nM (final concentration in the assay). Typically, the EC_{50} for human insulin is 0.06–0.10 nM. The insulin-like activity of compounds/drugs can be expressed as % of the maximal insulin stimulation (that is as the ratio between the fold stimulation provoked by the compounds/drugs with insulin-like activity at the maximally effective concentration and that induced by 5 nM insulin) to correct for the varying quality (i. e. insulin responsiveness) of the cells from different preparations.

K.6.1.2.2

Method Based on the Incorporation of a Fluorescent Fatty Acid Analog

PURPOSE AND RATIONALE

Usually lipogenesis is studied by the incorporation of radiolabeled glucose into toluene-extractable acyl-

glycerols in the presence of glucose at concentrations below 2 mM (see above). Under these conditions glucose transport and, in consequence, supply of glycerol-3-phosphate is rate-limiting. For uncoupling of glucose transport/glycerol-3-phosphate synthesis from (re)esterification, lipogenesis has to be performed in the presence of high glucose. To circumvent analysis of lipogenesis at high glucose (>2 mM) by (radio)labeling the glycerol moiety of TAG, which necessitates the application of glucose of high specific radioactivity, a fluorescent fatty acid analog can be used for its insulin-sensitive incorporation into TAG in isolated rat adipocytes.

Several recent reports suggest that the fluorescent fatty acid derivative, NBD-stearate, behaves like naturally occurring fatty acids, since it has been shown to enter rat hepatocytes by the same uptake mechanism as that described for unmodified fatty acids (Elsing et al. 1995, Storch et al. 1995). Furthermore, measurement of the uptake of a fluorescent long-chain fatty acid derivative into COS7 cells transfected with a 3T3-L1 adipocyte cDNA library led to the identification of fatty acyl-CoA synthetase and a putative fatty acid transport protein (Schaffer and Lodish 1994). Recently the efficient incorporation of NBD-FA, 12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoic acid, into mono- and diacylglycerol(-3-phosphate) in isolated rat adipocytes and its significant stimulation by insulin has been reported by Müller and coworkers (1997).

PROCEDURE

Incubation of the Adipocytes

200 μl of adipocyte suspension is supplemented with 190 μl of KRHB containing 0.1/2 mM glucose (=KRHB_{low}/KRHB_{high}) and 10 μl of an insulin solution (routinely 200 nM) or compound/drug candidate (routinely 200 μM in 20% DMSO) and incubated for 15 min at 37°C in a slowly shaking water bath. The assay is started by the addition of 50 μl of 0.9 mM NBD-FA (prepared daily from a 100 mM stock solution in ethanol by dilution with KRHB_{low}/KRHB_{high} under mild heating). After incubation at 37°C under mild shaking (stage 11, thermomixer, Eppendorff) for 90 min, lipogenesis is terminated by filtration of the total mixtures over GF/C-filters under vacuum. The filters are rapidly washed three times with 1 ml of KRHB_{low}/KRHB_{high} each, placed in 20-ml plastic scintillation vials and extracted with 400 μl THF for 15 min under rigorous shaking. 300 μl of the extract is transferred into new tubes and centrifuged (15,000 $\times g$, 5 min). The supernatant is dried (SpeedVac) and sus-

pended in 50 μ l THF. 5- μ l samples are analysed by TLC on silica gel Si-60 plates using 78 ml diethyl ether, 22 ml petrol ether, 1 ml acetic acid as solvent system. Fluorescent lipid products on the dried plates are visualized under UV (312 nm) and photographed (Polaroid CU5). The amount of acylglycerols (AG) is determined by fluorescence scanning with excitation at 342 nm and emission at 505 nm or fluorescence imaging with excitation at 460 nm and emission at 540–560 nm. The relative peak area of each lipid product minus a background value (derived from an equal-sized region of the TLC plate which does not contain any lipid product) is calculated as arb. units.

Characterization of the Fluorescent Lipids

For elucidation of the type of lipid(s), the synthesis of which is stimulated by insulin/compound/drug, the fluorescently labeled lipids are analyzed. This enables the characterization of the signaling and enzymatic steps preferentially activated during stimulus-induced lipogenesis. For this, adipocytes are labeled with 0.5 mM NBD-FA for 4 h at 37°C. Total lipids are extracted from the washed cells with chloroform/methanol (3/1 by vol.), dried and suspended in THF. NBD-fatty acylglycerol-3-phosphate, NBD-fatty acyl-palmitoylglycerol-3-phosphate and NBD-fatty acyl-palmitoylglycerol and NBD-fatty acyl-dipalmitoylglycerol are separated by TLC (Silica Gel Si-60 F₂₅₄) using toluene/ethylacetate (9/1 by vol.) as solvent system and subsequently eluted from the plate using the line elution method with ethylacetate as eluent. Larger amounts are separated by flash column chromatography (Still et al. 1978) on YMC-spherical silica (60 A, 40 μ M, YMC Europe GmbH, Schermbeck).

EVALUATION

The nitrobenzyl diazoly (NBD) moiety of NBD-FA is a strong fluorophore and is located far away from the carboxyl terminus. Furthermore, the apparent overall length of NBD-FA (carbon backbone including NBD moiety) is comparable to palmitic acid. It has been shown that insulin stimulation of the synthesis of the three lipid species harboring one NBD-fatty acyl residue each, in isolated rat adipocytes, closely resembles that of radioactive labeling of total lipids by [³H]glucose with regard to both insulin responsiveness and sensitivity. The data available suggest that incorporation of one residue of NBD-FA into glycerol-3-phosphate blocks further acylation, resulting in the accumulation of mono-NBD-fatty-acylglycerol-3-phosphate, NBD-fatty-acylpalmitoylglycerol-3-phosphate and NBD-fatty-acylpalmitoylglycerol rather than of

NBD-fatty acyl-dipalmitoylglycerol. One possibility is a stringent substrate specificity of diacylglycerol transferase (DGAT), for both the acceptor, diacylglycerol, and the donor acyl-CoA.

Stimulation of the rate-limiting enzyme of the esterification steps, GPAT, by insulin has been well established (Sooranna and Saggerson 1976, Vila and Farese 1991, Farese et al. 1994). It is generally accepted that in rat adipocytes the glucose transport step is rate-limiting for insulin-stimulated lipogenesis in the presence of 0.1 mM glucose in the incubation medium (see above). Thus under these conditions, the stimulation of synthesis of these AG by insulin may mainly reflect the effect of insulin on glucose transport. At 2 mM glucose, however, processes subsequent to glucose transport and glycerol-3-phosphate production will become rate-limiting. Both transport and esterification may be studied more conveniently using incorporation of NBD-FA rather than of [³H]glucose into lipids (see K.6.1.2.1), in particular under conditions of screening for compounds/drug candidates. Thus, assaying lipogenesis with NBD-FA can be used as a sensitive and reliable (non-radioactive) method for analyzing insulin signaling and lipid synthesis in rat adipocytes, in particular, if there is interest on the impact of high glucose concentrations in the incubation medium (and thus of conditions of insulin resistance; see K.6.3.5) on these processes.

K.6.1.3

Glucose Transport

GENERAL CONSIDERATIONS

Glucose transport is generally assumed to represent the rate-limiting step for lipogenesis in adipose tissue *in vivo* and in adipocytes *in vitro*, at least under conditions of low to moderate concentrations of glucose in the plasma and incubation medium, respectively (see above). Its stimulation by insulin is of exquisite sensitivity and responsiveness. Insulin resistance is defined as the reduced ability of cells or tissues to respond to physiological levels of insulin and is characteristic of non-insulin-dependent diabetes mellitus (type II diabetes). Skeletal muscle is the primary tissue responsible for the postprandial uptake of glucose from the blood in humans. The two major transporters expressed in adipose tissue/skeletal muscle are the muscle/fat-specific glucose transporter GLUT4 and the ubiquitous transporter GLUT1. The insulin-stimulated acute activation of glucose transport mainly occurs by one of two mechanisms: translocation of GLUT4 and GLUT1 from intracellular vesicles to the

plasma membrane and augmentation of the intrinsic catalytic activities of the transporters. The molecular mechanisms underlying the glucose transport and its regulation are similar for adipose and muscle cells. Consequently, adipocytes are more widely used for transport studies than myocytes due to their more convenient accessibility and more pronounced insulin responsiveness. However, studies for clarification of the mechanism by which compounds/drugs with insulin-like and/or insulin-sensitizing activity stimulate glucose uptake in skeletal muscle for the treatment of type II diabetes have to be performed with assay systems based on muscle cells or tissues. When differentiated into myotubes, cultured L6 muscle cells possess many of the properties of mature skeletal muscle tissue. They express both GLUT4 and GLUT1 and are capable of increasing glucose transport *via* insulin stimulation.

PURPOSE AND RATIONALE

Uptake of glucose in cultured cells is commonly determined by using non-metabolizable radioactive hexoses, such as 3-*O*-methylglucose (3MG) or 2-deoxyglucose (2DG), labeled with a high specific activity of tritium. [³H]3MG necessitates a very short incubation time due to fast equilibration of the analog across plasma membranes and requires either rapid separation of the cells from the aqueous incubation medium of the reaction mixture, usually by centrifugation through a suitable oil cushion with a buoyant density of less than 1, or the prevention of 3MG efflux by washing with a mercuric chloride solution. Uptake assays based on [³H]2DG are more convenient because 2DG is phosphorylated to a stable and membrane-impermeable derivative, 2-deoxyglucose-6-phosphate (2DG6P), by hexokinase or glucokinase, which may accumulate at a specific intracellular compartment, ensuring less rapid equilibration, slower kinetics and more convenient measurement of 2DG transport compared to 3MG (Frost and Lane 1985). However, routine use of these radiolabeled analogs is costly and requires a specialized institution where isotopes can be handled. Consequently, considerable efforts have been spent during the last decade in the development of assays using non-radioactive tracers, which are compatible with the analysis of glucose transport with sufficient insulin responsiveness.

K.6.1.3.1

Method Based on Radiolabeled 2-Deoxyglucose

Isolated Tissues

[2-³H]2DG (12 mCi/mouse) is injected i.p. into mice fasted for 6 h. After 40 min, mice are sacrificed and

tissues are excised and rinsed in ice-cold PBS, 1 mM EDTA. Tissue samples are homogenized in 0.5% perchloric acid and centrifuged. The protein pellet is solubilized in 0.3 N NaOH, 0.1% SDS for protein determination. The supernatant is neutralized with KOH and an aliquot is counted to yield total tissue counts (2DG and 2DGP). A second aliquot is treated with Ba(OH)₂ and ZnSO₄ to remove 2DGP and counted to yield [2-³H]2DG. Specific [2-³H]2DGP accumulation in tissues is calculated as difference between total tissue counts and [2-³H]2DG counts.

Primary and Cultured Adipocytes

50- μ l portions of adipocyte cell suspension (1×10^6 cells/ml) are pipetted into minisorp tubes (Nunc, Denmark) and equilibrated at 25°C for 30 min. Insulin (80 nM stock in KRH/5% BSA) and compounds/drugs (lyophilized, 2 mg/ml or 2% DMSO, 30 μ M) are added in 50 μ l KRHB and incubated for 30 min at 25°C. Thereafter, 50 μ l of 2-deoxy-D-[1-³H]glucose (2 μ Ci per ml of KRHB containing 10 μ l/ml of ³H-stock, 0.2 mCi/ml specific activity 20–30 Ci/mmol, 0.3 mM) are added (total volume of the final incubation mixture = 150 μ l). After further incubation for 20 min at 25°C in a shaking water bath, the assay is terminated by transfer of 100 μ l samples on top of 250 μ l dinonylphthalate (density < 0.93–0.96 g/ml, Merck, Darmstadt, FRG) in 500- μ l plastic tubes (Beckman) and centrifugation in a microfuge (10,000 $\times g$, 1 min, room temperature). The adipocytes remain on top of the oil layer, while the buffer is below the oil layer. The tube is cut with a special knife at the position of the oil layer below the adipocyte layer, then transferred into scintillation vials containing 5 ml aqueous scintillation cocktail (Zinsser Nr. 312 or Beckman Ready Safe) and counted for radioactivity. To correct for 2DG unspecifically associated with the adipocyte plasma membrane or entrapped in extracellular spaces or penetrated into the adipocytes by diffusion, 2 μ l of cytochalasin B (20 μ M final conc.) from a 1 mM stock solution (in 10% ethanol, diluted with H₂O from a 10 mM stock in ethanol at the day of use) and, as a control, 2 μ l of 10% ethanol are added prior to addition of radiolabeled 2DG and incubated under identical conditions (Gliemann et al. 1972, Foley and Gliemann 1981, Müller and Wied 1993).

EVALUATION

Specific transport is calculated as the difference between total cell-associated radioactivity (absence of cytochalasin B) and unspecifically associated/

entrapped radioactivity (presence of cytochalasin B). Under the conditions used, 2DG transport is linear with time up to 20 min. The fold stimulation for glucose transport is calculated as ratio between stimulated specific transport (presence of insulin/compound/drug) and basal specific transport (absence of insulin/compound/drug). For insulin (2 nM), this stimulation factor usually lies in the range between 15 and 25. To compensate for the varying insulin responsiveness of different adipocyte preparations, the insulin-like activity of compounds/drugs can be calculated as percentage of the maximal effect as described above for lipogenesis.

This assay measures the total glucose uptake encompassing the transport of the non-metabolizable glucose analog, 2DG, *via* the specific glucose transporters, GLUT1 and GLUT4, and the molecular mechanism of their movement from intracellular vesicles to the plasma membrane (GLUT translocation) including the underlying signaling cascade through which insulin stimulates glucose transport in adipocytes. Since 2DG taken up the cell is immediately phosphorylated by hexokinase, the assay actually monitors the accumulation of 2DG6P (presumably in specialized intracellular compartments). However, the rate-limiting step of glucose uptake at low glucose concentrations is the transport *via* GLUT1/4 rather than the phosphorylation. This results in apparently longer linear periods of glucose uptake in comparison to the use of non-phosphorylatable glucose analogs, such as 3MG (see K.6.1.3.3) and thereby considerably facilitates the experimental procedure and kinetic analysis. Subsequent conversion of the 2DG6P into lipids and glyco- gen or its oxidation does not occur. Thus, the use of 2DG is a reliable measure for glucose transport into adipocytes.

K.6.1.3.2

Method Based on Unlabeled 2-Deoxyglucose

PURPOSE AND RATIONALE

A non-radioisotope, enzymatic assay based on the methods of Manchester and coworkers (1990) and Sasson and coworkers (1993) for measuring of 2-deoxyglucose-6-phosphate (2DG6P) in tissues and cultured cells has been developed by Yamamoto and coworkers (2006). These methods enable the detection of 2DG6P accumulating in cells by measurement of the fluorescence of NADPH produced from NADP⁺, which is coupled to the oxidation of 2DG to 2DG6P by glucose-6-phosphate dehydrogenase (G6PDH). This approach, however, requires the culti-

vation of many cells on large plates and the preparation of cell extracts because the fluorescence of NADPH is rather weak. Consequently and more recently, a diaphorase–resazurin system that produces a potent fluorescent substance in the presence of NADPH has been linked. Uptake of 2DG into the cells can be measured by the addition of a single assay solution to the cell culture, followed by a simple incubation.

PROCEDURE

The differentiated 3T3-L1 adipocytes or L6 myotubes are incubated with 170 µl/well of α -MEM with 2% FBS in the presence of compounds/drugs/insulin for 10–30 min. After incubation, the cells are washed twice with Krebs–Ringer–phosphate–Hepes (KRPH) buffer (pH 7.4, 20 mM HEPES, 5 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 136 mM NaCl, 4.7 mM KCl) containing 0.1% BSA. The washed cells are then incubated with KRPH buffer containing 1 mM 2DG and 0.1% BSA for 1–2 h (termed the 2DG-uptake period) at 37°C in 95% O₂/5% CO₂. After incubation, the cells are washed twice with KRPH buffer containing 0.1% BSA and then 25 µl of 0.1 N NaOH was added. To degrade NAD(P)H, NAD(P)⁺ and any enzymes in the cells, the culture plate is subjected to one freeze–thaw cycle and incubated at 85°C for 40 min on a temperature-controlled bath. The components in the wells are then neutralized by the addition of 25 µl of 0.1 N HCl and then 25 µl of 150 mM triethanolamine (pH 8.1) is added.

To determine the low concentrations of 2DG and/or 2DG6P present in a 96-well microplate, a diaphorase–NADPH amplifying system is combined with previous methods (Sasson et al. 1993, Manchester et al. 1990) for measuring 2DG6P in tissues and cultured cells. 50 µl of 2DG solution at various concentrations is dispensed into each well of a 96-well plate and incubated for 90 min at 37°C after the addition of 150 µl of an assay cocktail of 50 mM triethanolamine (pH 8.1), 50 mM KCl, 0.5 mM MgCl₂, 0.02% BSA, 670 µM ATP, 0.12 µM NADP⁺, 25 µM resazurin sodium salt, 5.5 units/ml hexokinase, 16 units/ml G6PDH, and 1 unit/ml diaphorase. The assay cocktail is prepared before each assay from stock solutions of enzyme, coenzyme, and substrate that are maintained in the freezer or refrigerator. At the end of the incubation, fluorescence at 590 nm with excitation at 530 nm is measured by a microplate reader to detect the resorufin derived from reduced resazurin. A standard curve is generated by placing 2DG standard solutions in wells of the culture plate that had been prepared without cells.

EVALUATION

Glucose uptake in cultured cells is routinely determined by using non-metabolizable radioactive hexoses, such as 3MG or 2DG, labeled with a high specific activity of tritium. Assaying the uptake of [³H]2DG is more convenient than assaying the uptake of [³H]3MG because 2DG is converted to a stable and impermeable derivative 2DG6P through phosphorylation by hexokinase or glucokinase (see above). Because both methods rely on the use of substantial amounts of radioactive material, the concentration of the unlabeled analog is usually kept low (50–500 μM) in the uptake mixture to maintain a high specific activity. Measuring the uptake of these analogs at concentrations comparable to normal or pathological blood glucose levels (5–10 mM) is not practical because of the excessive amount of radioactive analog that would be required to maintain a specific activity high enough to obtain reliable data. Furthermore, the high concentration of analog needed is very expensive and a specialized institution registered for using radioactive isotopes is required. Although new fluorescently labeled glucose analogs for use as tracers of glucose uptake have been synthesized, none of these molecules has been found to show the same biological behavior as 2DG (Perret et al. 2004). Therefore, 2DG is often considered to be the gold standard in reference tracers of glucose transport and phosphorylation.

The resazurin–diaphorase-amplifying system facilitates the measurement of NADPH in the 2DG uptake assay, because the resorufin fluorophore produced is water-soluble and has high fluorescence intensity. In addition, there will be little background interference from other biochemical entities in the cell or from chemical compounds/drugs added during the assay because the emission wavelength of resorufin is longer and its fluorescence wavelength is much longer than those of NADPH. Furthermore, analysis of resorufin can be measured on standard, clear tissue-culture plates because the material comprising the culture plate, such as polystyrene, does not interfere with the fluorescence signal of resorufin.

K.6.1.3.3

Method Based on 3-*O*-Methylglucose

The rate of 3MG transport is determined according to Whitesell and Gliemann (1979), Karnieli and coworkers (1981), Basi and coworkers (1992) by a modification of the L-arabinose uptake method described by Foley and coworkers (1978).

PROCEDURE

5-ml polyethylene miniscintillation vials are prepared with 50 μl of incubation medium containing L-[1-³H]glucose and 3-*O*-[methyl-¹⁴C]glucose such that their concentration is 0.5 mM (58.2 and 11.6 μCi/μmol, respectively). L-Glucose is included as a marker for the extracellular space. Sugar uptake is initiated by the rapid addition of 200 μl of adipose cell suspension (2 × 10⁸ cell/ml) and then rapidly stopped by the addition of 10 μl of a 10 mM solution of cytochalasin B in 25% ethyl alcohol. The duration of this ‘pulse’ incubation is adjusted from 3 to 25 s so that the uptake achieved will be approximately one third of the equilibrium level. The equilibrium level of uptake is determined by incubating cells for 5 to 6 min in the presence of 3MG only and then for one additional min following the addition of L-glucose. Experimental blanks are obtained by adding cytochalasin B to the labeled sugar solution prior to the addition of cells. Following incubation, two 100-μl portions of each incubation mixture are separated from the incubation medium by centrifugation through dinonylphthalate, and the separated cells are counted for radioactivity (see above for 2DG transport). The net uptake of 3MG is then counted, and the initial uptake velocity or rate of transport is determined.

EVALUATION

3MG is a non-phosphorylatable glucose analog which can be used for the accurate measurement of the true initial glucose transport rate without interference with the subsequent glucose-metabolizing steps (e. g. phosphorylation to glucose-6-phosphate). Since the volume of the adipocyte cytosol is rather small, the time period required for equilibration of the extracellular and intracellular glucose concentration is rather short. This necessitates sophisticated experimental procedures enabling start and termination of the transport reaction within a few seconds. Therefore, for routine purposes 2DG rather than 3MG is commonly preferred.

K.6.1.3.4

Method Based on a Fluorescent Glucose Analog

Zou and coworkers (2005) described a sensitive and non-radioactive assay for direct and rapid measuring glucose transport in single living cells. The assay is based on direct incubation of mammalian cells with a fluorescent D-glucose analog, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) followed by flow cytometric detection of total fluorescence produced by the cells due to accumu-

lation of 2-NBDG-6-phosphate with time. However, despite a series of experiments has been conducted to define the optimal conditions for this assay, Zou and coworkers (2005) failed to demonstrate a clear-cut stimulation of 2-NBDG transport into these cells by insulin.

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K.6.1.4

Glucose Transporter Translocation

PURPOSE AND RATIONALE

The transport of glucose across the plasma membrane of mammalian cells represents one of the most important nutrient transport events, since glucose plays a central role in cellular energy homeostasis and intermediary metabolism. Apparently, rather than mediated by a single transporter molecule, glucose transport is mediated by a family of highly related transporters characterized by 12 transmembrane regions and amino-/carboxy-termini facing the cytoplasm (Assimacopoulos-Jeannet et al. 1991, Gould and Holman 1993, Mueckler 1994), the most important ones with regard to carbohydrate metabolism and regulation by insulin are:

GLUT1: the erythrocyte-type glucose transporter,

GLUT2: the liver-type glucose transporter,

GLUT3: the brain-type glucose transporter,

GLUT4: the insulin-responsive glucose transporter

GLUT5: the small intestine sugar/fructose transporter

GLUT8: a small intestine/hypothalamic glucose sensor and ion channel lacking a transport function

Among these isoforms, the insulin-responsive GLUT4 is the only one, which is expressed in mammalian muscle and adipose tissue, exclusively, and localized in cytoplasmic vesicles (GLUT4 vesicles) derived from the tubulo-vesicular structures of the trans-Golgi network in the unstimulated state. In animal and human adipose and muscle tissues insulin induces several-fold increases in glucose transport by causing the movement and fusion of GLUT4 vesicles to and with the plasma membrane, respectively. This so-called GLUT4 translocation leads to increase in the number of GLUT4 molecules exposed at the plasma membrane which results in elevated glucose transport velocity (i. e. increase in v_{\max}). Numerous cellular, animal and human studies have revealed significant impairment of GLUT4 translocation in the insulin-resistant and diabetic state. The molecular basis remains unclear so far but may include alterations in the complex machinery regulating the targeting, docking and fusion of the GLUT4 vesicles as well as in the phosphorylation of protein (SNARE) components involved (see below). This is suggested by the findings that GLUT4 is inactivated by phosphorylation in the insulin-resistant state and activated by dephosphorylation (Reusch et al. 1993) and that the phosphorylation state of GLUT4 is increased in primary rat adipocytes made insulin-resistant *in vitro* by incubation with high glucose and insulin (Müller and Wied 1993, see K.5.1.3).

K.6.1.4.1

Methods Based on the Determination of GLUT Molecules in Isolated Plasma Membranes

GENERAL CONSIDERATIONS

The insulin-stimulated GLUT4 translocation in adipocytes can be assayed by a number of different procedures either dependent on or independent of subcellular fractionation, i.e. the preparation of plasma membranes of high purity. This admittedly tedious and time-consuming procedure is prerequisite for the majority of techniques appropriate for the analysis of cell surface expression of GLUT4 (and thus reflecting GLUT4 translocation) in primary rat adipocytes, which had been challenged with insulin/compounds/drugs. These techniques rely on the determination of the amount of immunoreactive GLUT4 by immunoblotting with the recently introduced specific anti-GLUT4 antibodies in isolated

and sufficiently pure plasma membrane vesicles. Alternatively, radioactively or fluorescently labeled glucose analogs which are recognized but not transported by GLUT4 can be used for determination of the number of GLUT4 at the plasma membrane of intact rat adipocytes upon covalent crosslinking (see K.6.1.4.2.3). In contrast, in cultured murine or human adipocytes GLUT4 translocation can be followed upon transfection with a recombinant GLUT4 which is tagged with an extracellular epitope recognized by a specific antibody thereby circumventing the necessity for subcellular fractionation or covalent crosslinking (see K.6.1.4.1.2 and K.6.1.4.2).

Preparation of Rat Adipocyte Plasma Membrane Vesicles

Adipocytes are isolated from epididymal fat pads of 160–180 g male Wistar rats by collagenase digestion under sterile conditions and incubated with insulin or compounds/drugs as described (see K.5.1.2). The subcellular fractionation is performed according to McKeel and Jarett (1970) and Simpson and coworkers (1983) with the following modifications: The postnuclear supernatant is centrifuged ($12,000 \times g$, 15 min). The washed pellet is suspended in 35 ml of buffer and recentrifuged ($1000 \times g$, 10 min). The washed pellet ($12,000 \times g$, 20 min) is suspended in 5 ml of buffer, layered onto a 20 ml cushion of 38% (w/v) sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA and centrifuged ($110,000 \times g$, 60 min, 4°C). The membranes at the interface between the two layers (1 ml) are removed by suction, diluted with three volumes of buffer and layered on top of a 8 ml cushion of 24% Percoll, 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA. After centrifugation ($45,000 \times g$, 30 min) the plasma membranes are withdrawn with a Pasteur pipette from the lower fourth of the gradient (1 ml), diluted with 10 volumes of buffer and recentrifuged ($150,000 \times g$, 90 min, 4°C). The pellet is suspended, recentrifuged, finally dissolved in buffer at 2–5 mg protein/ml and stored at -80°C . The majority of plasma membranes are recovered as outside-out vesicles as revealed by the low cryptic activity of exofacial 5'-nucleotidase which is detected after detergent solubilization of the vesicles.

K.6.1.4.1.1

Determination by Immunoblotting

The methods described above lost their importance with the introduction of antibodies specific for GLUT4 representing the major (insulin)-regulated GLUT isoform which accounts for more than 90% of the max-

imal insulin-stimulated glucose transport activity in primary rat adipocytes and is localized in the basal state almost exclusively (95%) at intracellular vesicles and in the maximally stimulated state with 50% at the plasma membrane. Consequently, the amount of GLUT4 in plasma membrane vesicles derived from basal and stimulated (by insulin/compound/drug) rat adipocytes is now usually determined by immunoblotting for GLUT4. In case of weak expression of GLUT4 (e. g. cultured human adipocytes) or limited availability of starting materials (e. g. human adipose tissue), the sensitivity of the immunological detection can be considerably improved by immunoprecipitation of GLUT4 from the solubilized plasma membrane vesicles prior to immunoblotting.

Polyclonal affinity-purified rabbit antibodies have been successfully raised against a synthetic peptide corresponding to the COOH-terminal domain of rat GLUT4 (residues 495–509) (James et al. 1989). The plasma membrane vesicles are subjected to SDS-PAGE (25 µg protein/lane) and electrophoretically transferred onto nitrocellulose filters (Towbin et al. 1979). Incubations of the filters with primary and secondary antibodies (¹²⁵I-labeled or peroxidase-labeled anti-rabbit IgG from goat) and quantitative evaluation of the immunoreactive material by autoradiography and densitometry or enhanced chemiluminescence and luminescence imaging, respectively, are carried out as described previously (Müller and Wied 1993) for the demonstration of the sulfonylurea-induced GLUT4 translocation in isolated rat adipocytes, which may be based on phosphorylation of GLUT4. Evidence has been presented suggesting that insulin promotes serine phosphorylation of GLUT4 at the carboxyl terminus (Lawrence et al. 1990a, b) arguing for a causal relationship between GLUT4 translocation and phosphorylation in response to insulin and insulin-like acting sulfonylureas, such as glimepiride.

K.6.1.4.1.2

Determination of GLUT Molecules in Plasma Membrane “Sheets”

PURPOSE AND RATIONALE

In non-recombinant adherent murine and human adipocytes as well as non-adherent primary adipocytes which express endogenous GLUT4, only, its translocation can be monitored by the plasma membrane “sheet” assay. This is based on placing glass coverslips onto the adherent cells (6- or 12-well culture plates) following incubation with insulin/compounds/drugs. Upon removal of the coverslips the adherent cells are sheared and their plasma membranes attached to the

coverslip become disrupted, leaving the cytosolic components and the intracellular particulate materials (including the intracellular GLUT4 vesicles) with the medium of the plate. Subsequent immunofluorescent detection of GLUT4 remaining attached to the coverslips enables the semi-quantitative determination of GLUT4 located at the plasma membrane and apparently deprived from intracellular GLUT4.

PROCEDURE

The protocol for the preparation of plasma membrane sheets is derived from that of Moore and coworkers (1987) with modifications adapted from Robinson and coworkers (1992) and Fingar and coworkers (1993). 3T3-L1 adipocytes or human cultured adipocytes are plated on poly-D-lysine-coated coverslips the day before an experiment. Adipocytes are incubated for 1.5 h at 37°C in Leibovitz’s L-15 medium containing 0.2% BSA and treated in the absence or presence of insulin/compounds/drug candidates. The cells are washed twice in ice-cold buffer A (100 mM NaCl, 50 mM HEPES/KOH, pH 7.3) and once in buffer B (100 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 µM leupeptin, 2 µg/ml trypsin inhibitor, 0.5 mM benzamide, 20 mM HEPES/KOH, pH 7.2) and then sonified in buffer B for 3 sec using a Branson 250 sonifier with a microtip placed 1 cm above the coverslip (output control = 4, duty cycle = 30). Adherent plasma membrane sheets are washed twice in ice-cold buffer B, fixed in 3% paraformaldehyde for 10 min, and processed for indirect immunofluorescence using affinity-purified anti-GLUT4 antisera and rhodamine-conjugated secondary antibodies as described (Garcia de Herreros and Birnbaum 1989).

K.6.1.4.2

Methods Based on the Determination of GLUT Molecules at the Plasma Membranes of Intact Cells

GENERAL CONSIDERATIONS

For recombinant cultured murine and human adipocytes, the need for subcellular fractionation (i. e. preparation of plasma membrane vesicles of sufficient purity) can be circumvented by heterologous expression of GLUT4 tagged at the extracellular loop with an epitope for which specific antibodies are available (e. g. c-myc) and subsequent immunological detection of GLUT4 in the intact and appropriately fixed cells. Alternatively, GLUT exposed at the plasma membrane of intact adipocytes can be photoaffinity-labeled with membrane-impermeable specific and radioactive probes which enable the subsequent

detection upon immunoprecipitation of GLUT4/1 from total membranes. Moreover, the methods of “in-cell” immunoblotting or colorimetry enable accurate and straightforward detection of plasma membrane GLUT4 of cultured and subsequent to the treatment fixed adipocytes without the need for cell fractionation or plasma membrane sheet generation.

K.6.1.4.2.1

In-cell Immunoblotting

PURPOSE AND RATIONALE

The “in-cell immunoblot” is an immunocytochemical assay performed in microtiter plate format. Target-specific primary antibodies and infrared-labeled secondary antibodies are used to detect target proteins in fixed cells, and fluorescent signal from each well is quantified. Two targets at 700 and 800 nm using two spectrally distinct dyes are monitored. Separate lasers and fluorescence detectors are used for each dye and offer a wide linear detection range. With two detection channels two separate targets can be probed or the quantification accuracy is increased by using the second channel for normalization against a second target or DNA stain. Quantification accuracy is maximized by normalization because adjustments can be made for differences in cell number from well to well. Two-color normalization also helps to prevent false negatives and provides more accurate evaluation of the treatment of cells with compounds/drug candidates. The use of near-infrared probes yields high sensitivity for measuring small changes in the protein amount. The direct detection of proteins (e. g. GLUT4) in their cellular context eliminates variabilities and artifacts caused by cell lysis, gel loading, electrophoresis, and membrane transfer.

This method enables the analysis of cell surface expression of proteins (e. g. GLUT4) in adherent cells without the need for cumbersome and labor-intensive cell fractionation procedures. Upon incubation of the adherent adipocytes with insulin/compounds/drug candidates, GLUT4 expression at the plasma membrane is detected by “in-cell” immunoblotting using conditions for fixation and permeabilization of the adipocytes, which enable access of the anti-epitope antibodies to plasma membrane GLUT4, only, rather than to intracellular GLUT4 vesicles.

PROCEDURE

3T3-L1 fibroblasts or human adipocytes are seeded in 96-well clear round bottom plates (BD Bioscience cat# 353077) at 200,000 cells/well, grown to confluency and then differentiated into mature adipocytes as

described above. After serum deprivation overnight by replacement of the differentiation medium with serum-free DMEM, the adipocytes are incubated with insulin/compound/drug in 200 μ l of the same medium for 15 min at 37°C. For fixation, 25 μ l of 36% formaldehyde is added gently to each well using side of the wells (to avoid detaching of the cells from the well bottom). After incubation for 20 min at room temperature with very gentle rotation, the fixation solution is removed. For permeabilization, the cells are washed three times with 100 μ l of PBS containing 0.1% TX-100 for 5 min each by carefully pipetting the permeabilization solution down the sides of the wells to avoid detaching the cells from the well bottom. Thereafter, the permeabilization solution is removed and the washing step is repeated two more times. After addition of 100 μ l of blocking buffer (Odyssey, LICOR Biosciences) to each well and incubation for 1 h at room temperature with very gentle shaking on a rotator, 50 μ l of anti-GLUT4 antibody (1:200–1:500, Calbiochem) is added and the incubation continued overnight at 4°C. Thereafter, the primary antibody solution is removed and the plates are washed five times with 200 μ l of PBS containing 0.1% Tween-20 for 5 min each. 50 μ l of fluorescently labeled secondary antibody solution (goat anti-rabbit IRDye 800CW, Rockland Immunochemicals, 1:800 diluted in Odyssey blocking buffer with 0.2% Tween-20) is added to each well. After incubation for 1 h at room temperature on a rotator under protection from light, the secondary antibody solution is removed and the plates are washed five times with 200 μ l of PBS containing 0.1% Tween-20 at room temperature for 5 min each. After the final wash, the washing solution is completely removed from the wells. The plates are immediately scanned by placing on the Odyssey scan surface with detection in the 800 nm channel and at 169 μ m resolution at 3.0 to 3.5 mm focus offset and an intensity setting of 5 or less.

K.6.1.4.2.2

In-cell Colorimetry

PURPOSE AND RATIONALE

Understanding the molecular mechanisms of insulin action has been of major interest since the discovery, several decades ago, that insulin stimulates glucose transport *in vivo*. Subcellular fractionation (see K.6.1.4.1 and K.6.3.6.1), cell photolabeling coupled to immunoprecipitation and immunofluorescence (see below) or immunoelectron microscopy have been used to detect translocation of GLUT4 to the cell surface (Wilson and Cushman 1994, Kozka et al. 1991, Smith

et al. 1991). All of these methods are laborious and suffer from methodological inaccuracies. Subcellular fractionation is cumbersome and produces membranes that are rarely pure. Moreover, quantitative recovery of all membrane compartments is difficult or impossible. Affinity labeling of surface glucose transporters followed by immunoprecipitation depends on the ability to obtain quantitative immunoprecipitation and recovery upon SDS-PAGE. This technique results in the incorporation of about 1/10,000 of the label added, thus the signal to noise ratio is low. Moreover, the reactivity of the photolabel can depend on the level of activity of the transporter (Vannucci et al. 1992) in addition to the amount of transporter exposed at the cell surface. Immunofluorescence detection does not distinguish the native GLUT4 molecules incorporated into the cell membrane from molecules in subplasmalemmal vesicles, and is not a quantitative technique. Immunogold electron microscopy detects antigens at the plasma membrane accurately, but has not been successfully used in a quantitative fashion. None of the above techniques is suitable for large numbers of experiments, as would be required for screening of antidiabetic drugs stimulating insulin signaling or GLUT4 translocation. Therefore, Wang and coworkers (1998) intended to develop a fast and quantitative approach to measure GLUT4 translocation in intact cells. The method described uses cells in culture and does not require subsequent immunoprecipitation, SDS-PAGE or use of radioactivity, as required by the photolabeling technique. It does not require large amounts of cells nor laborious subcellular fractionation and is rapid, sensitive and quantitative.

PROCEDURE

Construction of L6 Cells Expressing c-myc Epitope-Tagged GLUT4 (GLUT4myc)

GLUT4myc cDNA is constructed by inserting the human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4 as described by Kanai and coworkers (1993). The epitope does not affect GLUT4 activity. GLUT4myc cDNA is subcloned into the mammalian expression vector pCXN (pCXN-GLUT4myc) as described by Niwa and coworkers (1991). L6 myoblasts are transfected with pCXN-GLUT4myc and pSV2-*bsr*, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride.

Cell Culture

L6-GLUT4myc myoblasts in cell monolayers are maintained in α -MEM supplemented with 10% FBS

in a humidified atmosphere containing 10% CO₂ and 90% air at 37°C. Cells are grown in 12- or 24-well plates and, prior to experiments, incubated with serum-free α -MEM supplemented with 25 mM glucose for 5 h.

Colorimetric Assay of Surface GLUT4myc

Quiescent L6-GLUT4myc cells are incubated with compounds/drug candidates, then washed once with PBS, fixed in 3% paraformaldehyde in PBS for 3 min at room temperature. The fixative is immediately neutralized by incubation with 1% glycine in PBS at 4°C for 10 min. The cells are blocked with 10% goat serum and 3% BSA in PBS at 4°C for at least 30 min. Primary monoclonal antibody (anti-c-myc, 9E10) is then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4°C. The cells are extensively washed with PBS before introducing peroxidase-conjugated rabbit anti-mouse IgG (1:1,000). After 30 min at 4°C, the cells are extensively washed and 1 ml *o*-phenylenediamine dihydrochloride reagent is added to each well. The colorimetric reaction is stopped by addition of 0.25 ml of 3 N HCl for 10 min at room temperature. The supernatant is collected and the optical absorbance is measured at 492 nm.

EVALUATION

Standard curves can be generated using either peroxidase conjugated anti-mouse IgG alone or myc-tag peptide. For this, myc-tag peptide at various concentrations is coated onto 24-well plates by incubation at 4°C for 24 h and then allowed to dry. The plates are rinsed with PBS to move excess salt and the uncoated spaces are blocked with 10% goat serum and 3% BSA.

K.6.1.4.2.3

Exofacial Labeling

PURPOSE AND RATIONALE

Alternatively, in non-recombinant primary and cultured adipocytes GLUT translocation can be analyzed by exofacial labeling of the GLUT molecules. For this, the membrane-impermeable photoreactive substrate analogs, radiolabeled bismannose propylamine (ATB-BMPA; Ryder et al. 2000) and biotinylated and spacer-equipped glucose compound (GP15; Hashimoto et al. 2001), which are all recognized but not transported by GLUT1 and GLUT4. In addition, a series of photoaffinity probes based on a dihexose structure, in which the hexose moieties (d-mannose or d-glucose) are linked through their 4-OH positions to a 2-aminopropyl spacer, to probe the cell-surface exposure of GLUT4 has been generated. Comparisons

of aryl azide, benzophenone and aryl diazirinetri-fluoroethane photoaffinity labelling derivatives of hexoses (Holman et al. 1986, 1988, 1990) have suggested that the latter are most suitable as they are highly efficient cross-linkers with good specificity. A (diazirinetri-fluoroethyl)benzoyl moiety has been attached through an amide link to the central amine group. More recently a biotinylated version of this compound has been developed in which a linker to biotin is attached through the hydroxy group of the 2-hydroxy-4-(1-diazirine-2,2,2-trifluoroethyl)benzoyl moiety (Koumanov et al. 1998). This compound has been used to assess the cell-surface exposure of GLUT4 in the human muscle of control and type II diabetic patients and marked impairments in the insulin-stimulated translocation of GLUT4 have been identified using this technique (Ryder et al. 2000). All these substrate analogs can be covalently crosslinked to the GLUT isoforms by photoaffinity-labeling *via* the attached diazirinetri-fluorobenzoyl moiety upon UV-irradiation following incubation of the cells with insulin/compounds/drugs. After removal of free substrate analog by washing of the adipocytes, total membranes are prepared from the lysed cells and counted for radioactivity (ATB-BMPA) or detected by immunoblotting for interaction with streptavidin (GP15). Both measures reflect the amount of total GLUT1/4 located at the plasma membranes at the time point of cell lysis, exclusively. For specific analysis of GLUT4 or GLUT1 translocation, the isoforms have to be immunoprecipitated with specific antibodies since the substrate analogs do not discriminate between GLUT4 and 1 (Holman et al. 1990).

PROCEDURE

For the use of photoaffinity labeling with GP15, (Hashimoto et al. 2001), primary rat adipocytes, which have been incubated with insulin/compounds/drugs are maintained at 18 C in the presence of 300–500 μ M photoreactive probe for 1 min either in the presence or absence of 0.5 M glucose. The samples are then irradiated for 1 min in a Rayonet photochemical reactor using 300 nm bulbs. Excess reagent is removed by extensive washing in physiological buffers and then 0.3 μ M avidin is added to block the cell-surface tagged transporters. Excess avidin is then removed by washing the cells. For detection of biotinylated GLUT4 by blotting, adipocyte samples are homogenised in HEPES/EDTA/sucrose (HES) buffer (255 mM sucrose, 1 mM EDTA, 20 mM HEPES (pH 7.2), 1 μ g/ml antipain, aprotinin, pepstatin and leupeptin, each, and 100 μ M 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). Homogenates are washed once

with HES buffer and subjected to centrifugation ($554,000 \times g$, 30 min, 4 C) to obtain a total membrane fraction. This pellet is solubilized in PBS, pH 7.2, with 2% of Thesit ($C_{12}E_9$) and 1 μ g/ml each of antipain, aprotinin, pepstatin and leupeptin, and 100 μ M AEBSF. The samples are solubilized for 50 min at 4 C with rotation and then subjected to centrifugation ($20,000 \times g$, 20 min, 4 C). Biotinylated proteins in the supernatants are either immunoprecipitated using a GLUT4 antiserum as described above or precipitated with streptavidin beads (Pierce, Rockford). Following GLUT4 immunoprecipitation, complexes are released into electrophoresis-sample buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol) at room temperature. The streptavidin precipitates are washed four times with PBS buffer containing 1% Thesit with protease inhibitors, four times with PBS containing 0.1% Thesit *plus* protease inhibitors and once in PBS. Electrophoresis-sample buffer is added to each pellet. The sample is then heated to 95 C for 30 min and subjected to centrifugation ($2,300 \times g$, 1 min). After removal of the supernatants, the pellets are washed with additional electrophoresis-sample buffer, heated to 95 C for 30 min and resubjected to centrifugation. Mercaptoethanol is added (10% final concentration) to the above samples in electrophoresis-sample buffer and these samples are then subjected to SDS-PAGE (10% gel). Proteins are transferred to nitrocellulose membranes. Membranes are blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween (TBS-T) and washed six times with TBS-T. Membranes are either incubated with streptavidin-HRP (Amersham) or with affinity-purified anti-GLUT4 C-terminal antibody in TBS-T containing 1% BSA (2 h, room temperature), followed by washing (six times in TBS-T) and detection using secondary antibody linked to horseradish peroxidase. GLUT4 protein is visualized with enhanced chemiluminescence (ECL).

K.6.1.4.3

Method Based on the Reconstitution of GLUT4 Translocation

PURPOSE AND RATIONALE

Insulin action on peripheral tissues including fat, heart, and skeletal muscle leads to increased glucose transport. The basis for this action lies in the increased translocation of GLUT4-containing vesicles from an intracellular reservoir compartment and ultimately exposure of the GLUT4 at the cell surface where it facilitates transport (see K.6.1.3 and K.6.1.4). Kinetic

studies have identified that GLUT4 traffics through multiple intracellular compartments involving endosomes, the trans-Golgi network (TGN), and a specialized compartment in which GLUT4 is enriched and it is the exocytosis process that is stimulated by insulin (Sato et al. 1993, Yang and Holman 1993). However, the site of insulin action along the exocytic pathway is unknown and potentially could involve increased release or budding of GLUT4 vesicles from tubular-vesicular structures, increased movements of vesicles along microtubules, increased transit through cortical actin near the cell surface, increased docking and then fusion of GLUT4 vesicles at the plasma membrane, and then possibly stimulation of catalytic activity of plasma-membrane-inserted GLUT transporters. For further resolution of the key-regulated step, an *in vitro* fusion reaction has been developed in which GLUT4 vesicles are immunisolated and fused with reconstituted plasma membrane in the presence of cell cytoplasm. The separate analysis of this partial reaction in the exocytic limb of the translocation sequence allows direct analysis of the extent to which fusion is stimulated by insulin or insulin-like compounds/drug candidates. Remarkably, the magnitude and time course for insulin's action on glucose transport is fully recapitulated in the vesicle fusion reaction.

Cell-free reconstitution of vesicle fusion reactions have many advantages for studying cell biology processes, as the separate components of the fusion reaction can be separately manipulated and combined. The cell-free approach is particularly powerful in studying insulin-regulated fusion as separate mixing of vesicles containing insulin-activated and non-activated components is feasible. With this approach, it can be studied whether the action of insulin/compounds/drugs leads to activation of GLUT4 vesicles, activation of components of the cytoplasm fraction, or activation of components of the plasma-membrane fraction. For insulin, recent data reveal that it is the activation of that plasma-membrane fraction that is the key-regulated step in the stimulation of fusion (Koumanov et al. 2005).

However, the exact nature of insulin signals that regulate GLUT4 translocation and how they affect the donor membrane, plasma membranes, or some other components in the translocation machinery remain unclear. In attempts to further characterize the translocation machinery and to study its modulation by insulin/compounds/drugs, two distinct *in vitro* reconstitution assays for GLUT4 translocation have been developed (Inoue et al. 1999, Koumanov et al. 2005).

K.6.1.4.3.1

Analysis by Immunoblotting

Preparation of Plasma Membranes and Donor Membranes

In an attempt to define the mechanism of insulin-regulated GLUT4 translocation, Inoue and coworkers (1999) developed an *in vitro* reconstitution assay based on immunoblotting detection of the membrane fusion reaction. For this, differentiated 3T3-L1 adipocytes (stably transfected with mycGLUT4 cDNA by using a retroviral expression plasmid for the preparation of donor membranes) in 10-cm dishes are serum-starved for 18 h and then incubated in the presence or absence of insulin/compounds/drugs. After washing twice with ice-cold PBS, the cells are homogenized immediately by using 20 strokes of a 1-ml Teflon-in-glass homogenizer in a buffer containing 10 mM HEPES/KOH (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 1 mM Na₃VO₄, 50 mM okadaic acid, 1 mM PMSE, and 0.1 mg/ml aprotinin. For preparing the donor membranes containing mycGLUT4, the homogenization buffer is supplemented with 0.5 mM ZnCl₂. The homogenate is centrifuged (500 × g, 1 min). For collection of plasma membranes, the supernatant is centrifuged (1,500 × g, 5 min). The supernatant is centrifuged (12,000 × g, 15 min). For collection of donor membranes (consisting of both low- and high-density microsomes), the supernatant is centrifuged (100,000 × g, 1 h). The resulting pellets of plasma membranes and donor membranes are suspended in the homogenization buffer without ZnCl₂, adjusted to a protein concentration of 2–4 mg/ml using the Bradford method and immediately used for the *in vitro* association assay.

In Vitro Association Assay

Donor membranes and plasma membranes (15–30 μg each) are mixed at 4°C in 75 μl of assay buffer (20 mM HEPES/KOH, pH 7.0, 250 mM sucrose, 0.5 mM EDTA, 1.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM DTT, 50 μg/ml BSA, 50 mM KCl), supplemented with an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 25 units/ml creatine phosphokinase). The amount of protein for plasma membranes and donor membranes in the assay is equal. The reactions are initiated by incubation at 37°C. After 2–10 min, the reactions are returned to 4°C and subjected to one of two procedures to separate the plasma membranes or plasma membrane-associated donor membrane vesicle structures from the donor membranes. Two different methods for the demonstration of the fusion of donor membranes and plasma membranes have been used successfully by Inoue and coworkers (1999).

Method I

The mixture is applied to the top of a discontinuous 28–44% sucrose gradient with 2% steps and subjected to centrifugation ($100,000\times g$, 18 h). Each 4-ml step layer is diluted with ice-cold water and centrifuged ($200,000\times g$, 1 h). The final pellets are dissolved in SDS-sample buffer.

Method II

The mixture is centrifuged ($15,000\times g$, 15 min) after dilution with 1 ml of the ice-cold homogenization buffer supplemented with 0.5 M KCl. The final pellet is dissolved in SDS-sample buffer. For detection of mycGLUT4 translocated from the donor membranes into the re-isolated plasma membranes during the *in vitro* association reaction, plasma membrane proteins are tested for the myc epitope by immunoblotting with appropriate primary antibodies and secondary anti-IgG antibodies coupled to peroxidase. The chemiluminescent signals for mycGLUT4 on the blot, which are quantitated by densitometric scanning have to be proportional to the load over the range used.

K.6.1.4.3.2**Analysis by Fluorescent Resonance Energy Transfer (FRET)****Isolation of Tagged GLUT4 Vesicles**

For demonstration of GLUT4 translocation in the reconstitution system introduced by Koumanov and coworkers (2005), rat adipocytes at 40% cytocrit are stimulated with 5 nM insulin for 20 min at 37°C. In a typical experiment 10 ml of cells are used. After cooling the cells to 18°C, the surface GLUT4 is labeled with 500 μ M biotinylated photolabel GP15 (see K.6.1.4.2.3) and incubated with 10 μ g/ml of cells DyLight647 streptavidin for a further 15 min. Insulin/compound/drug is removed and GLUT4 is internalized. The cells are then homogenized and processed as described by Simpson and coworkers (Simpson et al. 1983) to obtain a post-high-density-microsome supernatant, containing the fluorescently tagged GLUT4 vesicles. The GLUT4 vesicles are purified by immunoisolation using an anti-GLUT4 antibody (Satoh et al. 1993) prebound to the MBP-pa construct attached to a 0.7 ml amylose resin column. After 2 h incubation, the column is washed with intracellular buffer (IC buffer, 20 mM HEPES/KOH, pH 7.4, 140 mM potassium glutamate, 5 mM NaCl, 1 mM EGTA) and the GLUT4 vesicles are eluted in 500 μ l fractions with 20 mM maltose in IC buffer. The presence of GLUT4 in each fraction is monitored by measuring the DyLight 647 fluorescence. Usually, frac-

tions 1–3 are pooled together and used as a source of purified GLUT4 vesicles. GLUT4 in the fractions is analyzed using a sheep anti-GLUT4 antibody.

Preparation of Cytosol and Plasma Membrane-Liposomes

Concentrated cytosol is prepared from rat adipocytes in the basal state or following treatment with insulin/compounds/drugs for 20 min. Cells are washed once with buffer without BSA and once with IC buffer. As much buffer as possible is removed and the cells are vortexed and then spun ($265,000\times g$, 70 min). The cytosol is recovered between the pellet of crude membranes at the bottom and the fat layer at the top and kept frozen at -70°C until further use. Basal and insulin/compound/drug-treated plasma membranes are prepared (Simpson et al. 1983) and reconstituted in phospholipid-liposomes using an adaptation of previously described methods (Kono 1983, Schurmann et al. 1989). Purified plasma membrane is solubilized in 1.25% octyl glucoside and applied to Sephadex G50 column. 200- μ l aliquots of solubilized eluted protein (300 μ g/ml) are gently vortex mixed with 40 μ l of 150 mg/ml Soya bean phosphatidylcholine (Type IV-S, Sigma) in 20 mM HEPES (pH 7.4). A biotin-Europium (TMT) ligand is added at 55 nM final concentration and the mixture is sonicated for 15 s and frozen to -70°C for at least 2 h. The samples are then thawed, resonicated and purified on flotation Histodenz gradients. The liposomes are mixed 1:1 with 80% Histodenz in reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 1 mM DTT) containing 10% glycerol. This is overlaid with 30% Histodenz in reconstitution buffer containing glycerol, and then with buffer only. After centrifugation (35,000 rpm, 12 h, SW41Ti rotor), liposomes are collected at the 0/30% Histodenz interface. The amount of encapsulated biotin-Eu(TMT) is monitored by measuring the fluorescence against a standard curve. Typically 1–2 nM of Biotin-Eu(TMT) is encapsulated in 1 ml of isolated liposomes.

Fusion Assays

Fusion assays are performed in duplicate. 50 μ l of GLUT4 vesicles, liposomes and cytosol are mixed with 25 μ l of IC buffer containing 1 μ M biocytin on ice. Fusion is initiated by adding 50 μ l of ATP regenerating system (final concentrations: 1 mM ATP, 5 mM MgCl_2 , 8 mM phosphocreatine, and 31 U/ml creatine phosphokinase Type 1) at 37°C. In some cases GLUT4 vesicles and plasma membrane liposomes are mixed with the ATP regenerating system on ice and the reac-

tion is initiated by adding cytosol. Fusion is stopped by returning the samples on ice and addition of 1% TX-100 containing excess biocytin.

EVALUATION

The extent to which fusion had occurred prior to this endpoint is detected by TR-FRET between Europium (TMT) and DyLight 647 in a FARCyte microplate reader. The TR-FRET signal is calculated from the ratio of the emission at 670 nm/612 nm with excitation at 340 nm. The zero time point is used as a measure of the background and is subtracted from each value. Generally the extent of fusion at 5 min is compared with the maximal fusion obtained by incubation at 37°C for 30 min.

The *in vitro* reconstitution approach for GLUT4 translocation provides an unequivocal evaluation of the role of compounds/drug candidates in the fusion step as it represents a partial reaction in the GLUT4 vesicle translocation process. Although several recent microscopy studies on GLUT4 movement in cells have suggested that a regulated fusion step is likely (van Dam et al. 2005, Kanda et al. 2005, Lizunov et al. 2005), it is difficult to interpret such studies as many factors before and after the fusion step can influence the appearance of tagged-GLUT4 at the cell surface. Although the use of TIRF microscopy (Lizunov et al. 2005) has narrowed down the number of partial reactions involved in the insulin stimulation of exocytosis, the interpretation of these microscopy images is equivocal because the net incorporation of GLUT4 into the plasma membrane can be interpreted as indicating an involvement of the cytoskeleton, docking and fusion and endocytosis. In addition, fusion and endocytosis may be tightly coupled and the location of the membrane site at which these partial reactions occur may not be distinct. The *in vitro* analysis of the fusion reaction allows the fusion step to be studied in isolation from any involvement of the cytoskeleton or the endocytosis steps.

Applications

Li and McNeill (1997) reviewed various quantitation methods for the insulin-regulatable glucose transporter GLUT4 including reconstituted glucose transport, cytochalasin B binding assays, immunocytochemistry, immunoblots, ELISA, and exofacial labels. The effect of streptozotocin-induced diabetes on GLUT4 phosphorylation in rat adipocytes has been studied by Begum and Draznin (1992). The effect on glucose transporters by sulfonylureas was studied by Jacobs and Jung (1985), Jacobs and coworkers (1989),

that of metformin by Matthei and coworkers (1991). Cusin and coworkers (1990) found that hyperinsulinemia increases the amount of GLUT4 mRNA in white adipose tissue and decreases that of muscles. Hofmann and coworkers (1991) determined GLUT4 content by Western immunoblot protein analysis and by Northern blot analysis of GLUT4 mRNA in epididymal fat or soleus muscle tissue of streptozotocin-treated rats and of obese male KKA^Y mice after treatment with pioglitazone. The effect of *in vivo* thyroid hormone status on insulin signaling and GLUT1 and GLUT4 glucose transport systems in rat adipocytes was studied by Matthei and coworkers (1995). Galante and coworkers (1994) studied insulin-induced translocation of GLUT4 in skeletal muscle of insulin-resistant Zucker rats. Recycling of GLUT4 was studied by Laurie and coworkers (1993), Rampal and coworkers (1995). Bähr and coworkers (1995) studied the stimulation of myocardial glucose transport and glucose transporter-1 (GLUT1) and GLUT4 protein expression in cultured cardiomyocytes from rats. Teresaki and coworkers (1998) studied the influence of an insulin sensitizer on GLUT4 translocation in adipocytes of rats fed a high fat diet. Ren and coworkers (1995) evaluated the effect of increased GLUT4 protein expression in muscle and fat on the whole body glucose metabolism by the euglycemic hyperinsulinemic clamp technique in conscious mice. Uphues and coworkers (1995) used cardiac ventricular tissue of lean and genetically obese (fa/fa) Zucker rats to study the expression, subcellular distribution and insulin-induced recruitment of the glucose transporter GLUT4. Zeller and coworkers (1995) studied the GLUT1 distribution in adult rat brains using a newly developed immuno-autoradiographic method. Abe and coworkers (1997) reported molecular cloning of bovine GLUT4. Dalen and coworkers (2003) reported that the expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent in liver X receptor α .

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K.6.1.5 Fatty Acid Transport

PURPOSE AND RATIONALE

To gain knowledge of the biochemical pathways of fat metabolism in human physiology is central to understanding the derangements and pathology in the adipocyte handling of fatty acids associated with obesity and type II diabetes. The adipocyte stores large amounts of TAG, which continually turn over during lipolytic hydrolysis and reacylation of exogenous fatty acids. Human adipocytes do not have the capacity to synthesize significant amounts of fatty acids *de novo*. The large part of TAG synthesis comes from fatty acids provided by lipoprotein lipase hydrolysis of chylomicron or very low density lipoprotein TAG in the capillary blood vessels provided by the intestine and the liver, respectively. Fatty acids act as detergents that rapidly dissolve the plasma membrane, causing cell lysis if allowed to accumulate. More conflicting, the abnormal storage of TAG in the LD of cells from non-adipose tissues (muscle, liver, pancreatic β -cells) may interfere with the insulin regulation of lipid and carbohydrate metabolism as well as with glucose-induced insulin secretion *via* ill-defined molecular mechanisms which are collectively termed the lipotoxicity hypothesis for the pathogenesis of type II diabetes. The flow of fatty acids into the adipocyte can be high and these fatty acids have to be efficiently and rapidly converted to TAG and stored away in the cytoplasmic LD. The synthesis of lipids, in particular of TAG, including the biogenesis of LD by adipocytes depends on the uptake of free fatty acids (FFA) from the blood stream. FFA transport across the adipocyte plasma membrane involves both passive diffusion (at high FFA concentrations) and facilitated diffusion *via* specific transport proteins (at low concentrations). Current evidence indicates that several classes of fatty acid transport proteins, long-chain fatty acyl-CoA synthetases (ACS) and cytosolic fatty binding-proteins participate in fatty acid transport and activation. In eukaryotic systems, both fatty acid transport proteins (FATPs), fatty acid-binding proteins (FABPs), ACS and fatty acyl-CoA binding proteins are suggested to function in concert to facilitate long chain fatty acid uptake. There is considerable evidence that insulin stimulates fatty acid transport up to 3-fold in adipocytes by inducing the translocation of specific FATPs (CD36) from intra-

cellular vesicles to the plasma membranes, a molecular mechanism resembling GLUT4 translocation (see above). Potential drugs targeting these proteinaceous components of the transport pathway are promising. They may specifically block fatty acid uptake, cytoplasmic trafficking and/or activation in adipocytes for the pharmacotherapy of obesity, diabetes and the metabolic syndrome in humans. Conversely, stimulation of fatty acid uptake into adipocytes by potential drugs with insulin-like activity may be beneficial for the anti-diabetic therapy on basis of storage of excess of FFA in adipose tissue, the site specifically designed for the deposition of carbons taken in by daily food. The removal of FFA from the circulation would thereby circumvent the putative lipotoxic effects of FFA in other non-adipose tissues. It is hypothesized that high circulating levels of FFA occur when uptake and activation of fatty acids exceed the adipocyte's capacity for storage or β -oxidation. This leads to excess fatty acid internalization and resultant lipotoxicity of non-adipose cells and tissues (muscle, liver, pancreas). However, so far no drugs are available, which either increase or decrease the storage of TAG (by stimulation/inhibition of fatty acid transport and esterification or inhibition/stimulation of lipolysis in adipose tissue or non-adipose tissues, respectively). Their identification requires the development of assay systems for monitoring fatty acid transport, esterification and lipolysis appropriate for throughput screening.

K.6.1.5.1

Method Based on Radiolabeled Fatty Acids

Adipocytes

The [^3H]oleic acid uptake assays are performed with confluent 3T3-L1 adipocyte cell monolayers according to the procedure developed by Stremmel and coworkers (1986) and Pohl and coworkers (2005). [^3H]Oleic acid (173 μM , 0.05–0.20 μCi) is dissolved in a defatted BSA solution (173 μM) at a ratio of 1:1.2 ml of the oleate/BSA solution is incubated with the cell monolayer of each well of a 6-well culture dish at 37°C. The uptake is terminated after 5 min by removal of the solution followed by addition of 5 ml of an ice-cold stop solution (PBS containing 0.5% BSA and 200 μM phloretin). The stop solution is discharged after 2 min, and the culture dishes are washed by dipping them six times in ice-cold oleate/BSA solution (173 μM). NaOH (2 M) is added to lyse the cells. After neutralization, aliquots of the lysates are used for protein and radioactivity determination (in a liquid scintillation counter after addition of 10 ml of scintillation cocktail Ultima-Gold).

Hepatocytes

For measurement of fatty acid uptake into hepatocytes according to the procedure described by Chang and coworkers (2006), 200 μl of a suspension of freshly isolated hepatocytes (5×10^5 cells in PBS with >95% viability according to trypan blue exclusion) are added to 200 μl of oleate-BSA buffer solution containing 40 μM of oleic acid, 10 μM BSA and 5 $\mu\text{Ci/ml}$ [^3H]oleate in PBS. At increasing periods of time (0–120 s) afterwards, 5 ml of buffer containing 0.1% BSA and 500 μM phloretin in BSA are added to the mixture to stop the uptake. The entire mixture is immediately run through a glass fiber filter under low vacuum (<10 mm Hg) to collect the cells. The glass fiber is placed in a vial with scintillation fluid to count radioactivity. A blank vial and a vial with an aliquot of oleate-BSA buffer solution is used as negative and positive controls, respectively. The assays done at the time point zero are used as the background uptake activity and subtracted from the activity measured at each time point. The final values are corrected for cell viability, determined after isolation of the hepatocytes by trypan blue exclusion or MTT.

Viability of Cultured Cells – MTT Assay

To evaluate the cytotoxic effect of the drugs, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is performed. Cells are seeded in 12-well plates and incubated with compounds/drug candidates. Subsequently, 200 μl of 0.25% (w/v) MTT is added to each well, and cells are further incubated for 2 h. The resulting formazan crystals are then solubilized by adding 1 ml of MTT lysis solution (10% SDS and 1 mM acetic acid in DMSO). Absorbance at 570 nm is measured. The results are expressed as the percentage of absorbance related to control cells.

Viability of Cultured Cells – Trypan Blue Assay

After incubation of the cells in the absence (control group) or presence of compound/drug candidate, the cells are exposed to the membrane-impermeant dye, trypan blue (0.1%, w/v) for 15 min at 37°C. The presence of dye is determined by light microscopy and the numbers of unstained and stained cells in the field are counted to obtain an estimate of the percentage of the cells taking up the dye.

Viability of Cultured Cells – Lactate Dehydrogenase (LDH) Release

After incubation of the cells with compound/drug candidate, LDH release into the medium is measured by

determining LDH activity (cytotoxicity detection kit-LDH, Roche Applied Science). The amount of color formed in the assay is proportional to the number of lysed cells. The LDH activity in the total of dead islet cells (high control) is measured after solubilization of the cells with 5% (by vol.) Triton X-100.

EVALUATION

To determine the percentage cytotoxicity, the absorbance at 490 nm is measured in multiple samples with subtraction of values obtained in control incubation (low control with cells but without compound) using the following equation:

$$\text{Cytotoxicity(\%)} = \frac{\text{compound value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

K.6.1.5.2

Method Based on Fluorescent Fatty Acids

GENERAL CONSIDERATIONS

Fatty acid transport can be more easily studied in yeast compared to mammalian cells, which makes a yeast-based assay system attractive for throughput screening in drug discovery. Fatty acid uptake has been demonstrated to be essential for growth of yeast under conditions of blocked *de novo* synthesis. This occurs in the natural state when cells are grown under hypoxic conditions due to the requirement of the acyl-CoA desaturase for oxygen. During hypoxia, cells specifically require an exogenous source of long-chain unsaturated fatty acids. Fatty acid uptake is dependent upon two proteins, Fat1p, the orthologue of the mammalian FATPs and an ASCL, either Faa1p or Faa4p. Yeast with deletions in the structural gene encoding Fat1p (FAT1) or Faa1p and Faa4p (FAA1 and FAA4) cannot grow anaerobically whether or not unsaturated fatty acids are added to the growth media due to a defect in fatty acid activation (Faergeman et al. 1997 and 2001, Dirusso et al. 2000). These deficiencies in transport and growth can be alleviated by expression of a selected group of FATPs (mmFATP1, mmFATP2 and mmFATP4; Johnson et al. 2000). The yeast system was recently successfully exploited as a method for screening fatty acid uptake inhibitors, which function by specific interaction with a mammalian FATP conditionally expressed in yeast (Li et al. 2005).

PURPOSE AND RATIONALE

This method (Li et al. 2005) is based on the use of the fluorescent fatty acid analog, 4,4-difluoro-5-methyl-4-

bora-3*a*,4*a*-diazas-indacene-3-dodecanoic acid (C₁-Bodipy-C₁₂). The length of C₁-Bodipy-C₁₂ is approximately equivalent to that of an 18-carbon fatty acid. The Bodipy fluorophore is 11 carbons away from the carboxylate group. Both a non-homogenous and homogenous assay designs have been developed. Cellular uptake of C₁-Bodipy-C₁₂ can be monitored using fluorescence microscopy. For this, cells are first incubated with C₁-Bodipy-C₁₂ to allow uptake and then unincorporated ligand is removed using repeated washes with the fatty acid-binding protein BSA. For detection of fatty acid uptake, the cells are attached to microscope slides by coating with polylysine and then visualized using confocal microscopy. For high-throughput screening, this is not practical, however, due to the requirement of several washing cycles and centrifugations or of microscopy as detection method. The homogenous assay avoids multiple washing steps and relies on a standard microtiter plate fluorescence reader. In this method, extracellular fluorescence is quenched by the addition of trypan blue which does not penetrate into intact 3T3-L1 adipocytes. The assay has been validated with the non-fluorescent natural fatty acid, oleic acid, in addition to sodium azide and Triacsin C, compounds that interfere with fatty acid uptake by blockade of the fatty acid activation step.

PROCEDURE

Yeast cells with the genes for Fat1p and Faa1p deleted and transformed with the expression vector encoding murine FATP2 (mmFATP2) under the control of the GAL10 promoter are grown to 0.1 A₆₀₀ (mid-log phase) in yeast-supplemented minimal media containing 2% galactose and 2% raffinose (without leucine and histidine) at 30°C to induce expression of the mmFATP2. Cells are harvested by centrifugation (5,000×g, 5 min) and then resuspended in PBS at a cell density of 6 × 10⁷/ml prior to use. In a standard uptake assay, 50 μl of yeast cells (3 × 10⁶) is dispensed into each well and incubated with 5 μM C₁-Bodipy-C₁₂ in the dark for 3 min in 100 μl total volume. C₁-Bodipy-C₁₂ is prepared in PBS containing BSA (7.5 μM final concentration in the assay). Then, 50 μl of trypan blue (0.33 mM final concentration) is added to the reaction to quench the extracellular fluorescence of C₁-Bodipy-C₁₂. Fluorescence intensity is measured on a fluorescence microtiter plate reader (e.g. Molecular Devices) with filter set at 485 nm excitation and 528 nm emission. Fluorescence intensity is recorded after adding trypan blue at room temperature for various times (0, 30, 60 min). The ratio of measured fluorescence intensity of yeast cells expressing

and lacking mmFATP2 is calculated and used to assess the C₁-Bodipy-C₁₂ uptake activity of the corresponding mammalian FATP. Cells are incubated with compounds/drugs on an orbital shaker (250 rpm, 30°C) for 2 h prior to assaying C₁-Bodipy-C₁₂ uptake.

EVALUATION

This yeast-based assay system introduced by Li and coworkers (2005) for the identification of compounds/drug candidates that target the mammalian FATPs is critically dependent on the bypass of the time-consuming repeated washing steps with BSA required to limit the fluorescent signal from unincorporated ligand. For this, a quenching reagent was chosen, that decreases the signal of non-cell-associated C₁-Bodipy-C₁₂, which obscures the cell-associated fluorescence. Consequently, the quenching dye must have an absorbance spectrum that significantly overlaps the fluorescence emission of the Bodipy fluorophor, such as Trypan blue, with a peak absorbance at 607 nm. This dye has been shown to quench the extracellular fluorescent probes used in phagocytosis assays but does not quench internal fluorescence since it is effectively excluded from living cells due to impermeability of the cell membrane to this compound. BSA is added to the assay mixture to solubilize the hydrophobic fatty acid analog. It has been found that the apparent fluorescence measured in the presence of BSA is much more consistent compared to that measured in the absence of BSA. The signal difference between the positive and the negative controls (ratio of cells expressing and lacking the mammalian FATP) is usually in the range of 4- to 5-fold. Therefore, utilizing trypan blue to quench the non-cell associated C₁-Bodipy-C₁₂ it is possible to omit the washing cycles for monitoring fatty acid uptake.

One advantage of the yeast cell-based assay to target the mammalian FATPs is that it relies on a phenotype (i. e. fluorescence signal) specific to the target protein. Thus, it mimics the biological context of the protein more closely than the cell-free system (Fernandes 1998). A draw-back of the cell-based assay, however, is the lack of knowledge about the mechanism of interaction with and specificity toward the target protein. Therefore, secondary screens must be performed, which are required to eliminate toxic compounds. Compounds which kill the cells allow the non-specific entry of trypan blue leading to quench of the internalized ligand. Additionally, some selected compounds might work by non-FATP-mediated mechanisms, including those that increase the permeability of the cell membrane and facilitate trypan blue entry

into the cells. In conclusion, the data presented so far demonstrate that the yeast cell-based fatty acid uptake assay employing a *S. cerevisiae* strain deficient in fatty acid uptake, that expresses mmFATP2 and tests the mmFATP2-dependent C₁-Bodipy-C₁₂ transport activity can be used for high-throughput screening of inhibitors or activators of the fatty acid transport and may be successfully applied to other members of the FATP and ACS families.

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K.6.1.6 Cellular Esterification

PURPOSE AND RATIONALE

The following assay monitors the complete pathway of esterification of glycerol-3-phosphate synthesized *via* glycolysis from glucose, which is transported into the adipocytes upon insulin-dependent GLUT4 translocation with fatty acids taken up by the adipocytes *via* the fatty acid transport proteins (in insulin-dependent fashion) (see K.6.1.2 and K.6.1.5).

PROCEDURE

For this assay described by Sooranna and Saggeron (1976), adipocytes are treated with low concentrations of saponin to permeabilize the plasma membrane (without disrupting internal membranes) and subsequently incubated with L-[U-¹⁴C]glycerol-3-phosphate for 10 min at 25°C. Toluene-based scintillation cocktail is added and the lipid and aqueous phases are separated by centrifugation. The lipid phase is removed and counted for radioactivity.

EVALUATION

This assay measures the complete esterification pathway of glycerol-3-phosphate into lipid-soluble products (TAG, phospholipids, lipidic intermediates such as [lyso]phosphatidic acid) including the regulatory cascade which is stimulated by insulin. One or the other of the activation or acylation steps may be affected by compounds/drug candidates. Glucose transport does not interfere due to the use of the permeabilized adipocytes as is revealed by lack of inhibition of basal esterification by cytochalasin B.

K.6.1.7**Lipid-Synthesizing Enzymes****PURPOSE AND RATIONALE**

The effects of insulin/compounds/drugs on the three acylating enzymes involved in the esterification pathway, glycerol-3-phosphate-acyltransferase (GPAT), monoacylglycerol-3-phosphate-acyltransferase (AGPAT) and 1,2-diacylglycerol-acyltransferase (DGAT) (for a review see Coleman and Lee 2004) can be assayed individually using microsomes (GPAT, AGPAT, DGAT) and mitochondria (GPAT, AGPAT) prepared from adipocytes which have been pretreated correspondingly. Care must be taken to preserve the activation state of the acylating enzymes during preparation of the microsomes after challenge of the adipocytes with insulin/compounds/drugs by the inclusion of mixtures of phosphatase and protease inhibitors. For the analysis of putative direct effects of compounds/drugs on the acylation enzymes, they are added to the cell-free incubation mixtures containing microsomes/mitochondria from untreated adipocytes.

Preparation of Microsomes/Mitochondria

Isolated rat adipocytes, which have been incubated with insulin/compounds/drugs, are homogenized by 10 strokes in a motor-driven Teflon-in-glass homogenizer in TES buffer (20 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA) supplemented with 10 μM

okadaic acid, 10 mM sodium pyrophosphate, 100 mM NaF, 10 mM glycerol-3-phosphate, 1 mM sodium orthovanadate, 0.1 mM PMSF, 10 μg/ml each of antipain, leupeptin, pepstatin and E-64. After differential centrifugation steps for the removal of cell debris (500 × g, 5 min), plasma membranes (2,000 × g, 15 min) and mitochondria (10,000 × g, 20 min), the supernatant is centrifuged (100,000 × g, 60 min, 4°C). The pellet is resuspended in TES buffer and centrifuged (16,000 × g, 10 min). The microsomes are recovered from the supernatant by centrifugation (100,000 × g, 60 min, 4°C). Microsomes and mitochondria are resuspended in TES buffer (at 4 mg protein/ml) and finally stored at -80°C and used only once after thawing. According to the distribution of the marker enzymes (NADPH-cytochrome-c reductase, GRP-78 and BiP for microsomes, succinate dehydrogenase and porin for mitochondria, adenylate cyclase and Na⁺/K⁺-ATPase for plasma membranes), the microsomes are contaminated to varying degree with mitochondria and are significantly deprived of plasma membranes.

K.6.1.7.1**Glycerol-3-Phosphate Acyltransferase (GPAT)****PROCEDURE****Radioactive Assay**

The incubation mixture contains 50 mM Tris/HCl (pH 7.4), 200 mM KCl, 1 mM DTT, 150 μM palmitoyl-CoA, 4 mM MgCl₂, 10 mM NaF, 2 mg/ml BSA, 0.2 mM [³H]glycerol-3-phosphate (2 μCi) and microsomes from primary rat adipocytes (25–100 μg) in a final volume of 0.5 ml. The reaction is started by addition of the microsomes in the absence or presence of 2 mM *N*-ethylmaleimide (NEM). After incubation (3 min, 37°C), the reaction is stopped with 2 ml of water-saturated butanol, followed by 1.5 ml of butanol-saturated water. The butanol phase is separated and washed twice. An aliquot is counted for radioactivity. Under these conditions the rate of product formation is linear for up to 15 min.

Fluorescent Assay

Up to 100 μl of microsomes/mitochondria are incubated with 100 μl of reaction mixture containing 40 mM Tris/HCl (pH 7.4), 8 mM MgCl₂, 4 mg/ml BSA, 10 mM NaF, 1 mM NDC, 0.3 mM glycerol-3-phosphate in a total volume of 200 μl. After incubation (60–180 min, 25–30°C) in the absence or presence of 2 mM NEM, the reaction is terminated by the addition of 300 μl of chloroform/methanol (2/1, by vol.) containing 0.1 M HCl and thoroughly mix-

ing. After phase separation ($5000 \times g$, 10 min), 180 μ l of the lower organic phase are removed and dried. The resin is solubilized in 50 μ l of dichloromethane by thorough mixing. Portions (5 μ l) are analyzed by thin layer chromatography (Merck, silicagel Si-60) using acetic acid methylester/toluol (2/1 or 1/1, by vol.) with synthetic NBD-labeled mono-, di- and tridodecanoyl-glycerol run in parallel as markers.

EVALUATION

In case of using of microsomes which are equipped with the complete lipid-synthesizing machinery, the predominant radiolabeled/fluorescent product formed is TAG with accumulation of only minor amounts of its precursors, phosphatidic acid (3–8%) and lysophosphatidic acid (1–4%) as determined by thin layer chromatography and fluorography. However, under the conditions used GPAT activity is rate-limiting for the formation of radiolabeled or fluorescent TAG and consequently, the rate of TAG formation is a direct reflection of the activity of GPAT. In case of using mitochondria, which are typically contaminated by microsomes, their total GPAT activity is determined in the absence of NEM. mtGPAT1 activity, which is resistant to inhibition of NEM (Bell and Coleman 1980), is determined in the presence of 2 mM NEM and subtracted from the total activity to yield the NEM-sensitive GPAT activity, representing the activity from the microsomal GPAT and mtGPAT2 (Bell and Coleman 1980, Lewin et al. 2004). Typically, 2 mM NEM is sufficient to inhibit all of the microsomal GPAT and mtGPAT2 activity.

Time- and concentration dependence of sulfonyl-urea-stimulated GPAT was tested by Müller and coworkers (1994). Insulin has been reported to stimulate microsomal GPAT by 2- to 3-fold in primary rat adipocytes. The molecular mechanism of this regulation remains to be elucidated. Phosphorylation (by casein kinase II and allosteric activators (polar head groups derived from glycosyl-phosphatidylinositol lipids) have been discussed (Vila et al. 1990; see K.6.3.6.4).

K.6.1.7.2

Acylglycerol-3-Phosphate Acyltransferase (AGPAT)

PROCEDURE

Radioactive Assay

1-ml incubation mixtures are prepared by sonication of monoacyl-glycerol-3-phosphate (MAGP) (up to 100 μ M), TX-100 (0.5%), phosphatidylcholine (0.2 mg/ml), [14 C]palmitoyl-CoA (40 μ M),

NaF (50 mM), MgCl₂ (1.5 mM) in 150 mM Tris/HCl (pH 8.0). The reaction is started by addition of up to 20 μ g of microsomes. After incubation (10 min, 37°C, constant shaking), the reaction is terminated by the addition of 4.5 ml of chloroform/methanol (1/2, by vol.), then of 1.5 ml of chloroform and 1.5 ml of 2 M KCl/0.2 M H₃PO₄. Diacylglycerol-3-phosphate contained in the chloroform layer is dried, redissolved in chloroform/methanol (2/1, by vol.) and then analyzed by TLC using chloroform/methanol/acetic acid/water (25/10/3/1, by vol.). Dried TLC plates are subjected to fluorography (ENHANCE spray, NEN/DuPont; Kodak X-Omat AR films).

Spectrophotometric Assay

AGPAT can be assayed by measuring the reaction of released CoA-SH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB = Ellman's reagent) spectrophotometrically. The reaction mixture consists of 100 mM Tris/HCl (pH 7.4), 20 μ M oleoyl-CoA, 50 μ M 1-acylglycerol-3-phosphate, 1 mM DTNB (10 mM stock in 1 M Tris/HCl, pH 8.0) and up to 50 μ l of sample (~200 μ g microsomal protein) in the presence of compounds/drug candidates in the wells of a 96-well microtiter plate (200 μ l total vol.). The reaction is initiated by the addition of oleoyl-CoA after pre-incubation of the microsomes with all other components for 2–4 min. The increase in absorbance at 405 nm is followed at 37°C with a microtiterplate spectrophotometer. Control incubations are performed in the absence of 1-acylglycerol-3-phosphate, and the observed rates are subtracted from the rates measured in the presence of acyl acceptor.

K.6.1.7.3

Diacylglycerol Acyltransferase (DGAT)

PROCEDURE

The incubation mixture (250 μ l) contains 150 mM Tris/HCl (pH 8.0), 10 mM MgCl₂, 1 mg/ml BSA, 50 μ M [14 C]palmitoyl-CoA (3 μ Ci, 25,000 cpm/nmol). The reaction is started by the addition of up to 50 μ l of a mixture containing 1.5 mM 1,2-diacylglycerol and phosphatidylcholine (0.2 mg/ml) in absolute ethanol and up to 100 μ g of microsomal protein. After incubation (15 min, 37°C), the reaction is terminated by the addition of 1 ml of 2-propanol/heptane/water (80/20/2, by vol.). TAG contained in the heptane layer is further purified. The dried samples are redissolved in chloroform/methanol (2/1, by vol.) and analyzed by TLC using petroleum ether/diethyl ether/acetic acid (80/20/1,

by vol.) or hexan/diethylether/acetic acid (70/291/1, by vol.) and fluorography or radioactivity scanning.

K.6.1.8

Formation of Lipid Droplets (LD)

GENERAL CONSIDERATIONS

In vertebrate animals the most abundant energy reserve is stored as TAG in the LD of adipocytes. These LD can be as large as 100 μm and are composed of a core of TAG surrounded by a phospholipid and cholesterol monolayer into which numerous proteins are embedded (DeLany et al. 2005, Brasaemle et al. 2004, Wolins et al. 2005, Robenek et al. 2004, 2005a, 2005b). Most other types of cells contain tiny LD that store primarily cholesterol esters and serve as a reservoir of cholesterol for the synthesis and maintenance of membranes. Steroidogenic cells of the adrenal cortex, testes, and ovaries use stored cholesterol additionally as a source of substrate for steroid hormone synthesis. Little is known about the mechanisms that control the flux of neutral lipids into and out of LD in any type of cell, but it is clear that the processes that control lipid traffic in adipocytes are central to the regulation of whole body energy metabolism. Moreover, the biogenesis of these structures, in general, and in adipocytes, in particular, remains to be elucidated. Several proteins have been identified on their surface, including adipocyte differentiation-related protein (ADRP or adipophilin), perilipins, and caveolin, but their roles in LD assembly are unknown.

ADRP and perilipins are abundant on LD. ADRP is found mostly on smaller LD. Caveolin is a 21-kDa membrane protein with a hairpin structure whose N and C termini face the cytosol. This is in agreement with the model proposed for the assembly of LD. According to this model the TAG will during the biosynthesis “oil-out” between the leaflets of the bilayers, forming lens-shaped structures in the membrane. The LD, covered by a monolayer, will be formed by the budding of such a lens from the membrane. The structure will be stabilized by proteins bound to both the luminal and cytosolic surface of the membrane.

PURPOSE AND RATIONALE

Cell-free systems are extremely useful for studies of sorting processes, such as the budding of transport vesicles and are the only method for determining the mechanism by which such complex structures are formed. A microsome-based, cell-free system has been developed by Marchesan and coworkers (2003) to study the transport of TAG from microsomes to the cy-

tosol. This system assembles TAG-containing LD similar to the small LD isolated from 3T3-L1 cells. Caveolin, ADRP, vimentin, and the GRP-78 are present on these LD and the release of the LD from microsomes is dependent on PLD and the formation of PA. This cell-free system may be helpful for the screening and characterization of compounds/drug candidates activating or inhibiting LD biogenesis by interference downstream of TAG synthesis (e. g. DGAT activity, assembly of LD-associated proteins at the ER, LD release from the ER) as well as for the elucidation of the mode of action of compounds/drug candidates identified in cell-based assays for lipogenesis (see K.6.1.2).

K.6.1.8.1

Preparation of Microsomes

3T3-L1 cells are cultured and differentiated for 2 days (unless stated otherwise) as described above. The cells (2×10^6 cells/incubation) are homogenized (30 strokes in a Dounce glass homogenizer in 10 mM Tris/HCl (pH 7.5) containing 250 mM sucrose, 1 mM EDTA, 100 KIU/ml Trasylol, 0.1 mM leupeptin, 0.1 μM pepstatin, and 1 mM PMSF, and the microsomes are isolated by two distinct gradient ultracentrifugation methods as described (see K.6.1.8.2). To remove loosely bound (non-integral) proteins from the microsomes, the pellet is resuspended in 500 mM Tris/HCl (pH 7.5) containing 1.2 M KCl (high salt wash) and incubated (30 min, 4°C) with head-over-tail mixing. The microsomes are then recovered by ultracentrifugation (160,000 $\times g$, 70 min, 12°C). The washed microsomes are resuspended in 10 mM Tris/HCl (pH 7.5) containing 250 mM sucrose.

K.6.1.8.2

Cell-Free System

Incubation

The *in vitro* system used to investigate the formation of LD and introduced by Marchesan and coworkers (2003) is based on microsomes that had been subjected to a high salt wash, an activator from the 160,000 $\times g$ -supernatant from homogenized rat adipocytes, and a substrate for DGAT. To prepare the DGAT substrate, 0.25 mg of 1,2-diacylglycerol is dissolved in 40 μl of 10 mM Tris/HCl (pH 7.5) with 1.0 mM palmitoyl-CoA by gentle vortexing: 1.25 μl of [^{14}C]palmitoyl-CoA (55 mCi/mmol; total 0.06 μCi) and 360 μl of 10 mM Tris/HCl (pH 7.5) are added, and the mixture is vortexed again. To start the reaction, 600 μl of the partially purified cytosolic activator in 10 mM Tris/HCl

(pH 7.5) containing 250 mM sucrose, 150 μ l of 1.4 M $MgCl_2$ (in the same buffer), and 50 μ l of the microsome solution (0.5–1 mg of microsomes) are added to the DGAT substrate. Incubation is carried out (37°C, 60 min) (the production of LD plateaued between 30 and 60 min). After the incubation, the total production of radioactive TAG is measured, and the incubation mixture is subjected to gradient ultracentrifugation as described below. Before centrifugation, the mixture is supplemented with (final concentrations) Trasylol (100 KIU/ml), leupeptin (0.1 mM), PMSF (1 mM), pepstatin A (1 μ M), N-acetyl-Leu-Leu-norleucinal (5 μ M), and EDTA (0.5 mM).

Gradient Ultracentrifugation

For gradient I, the samples are adjusted to 25% sucrose, and 1 ml is layered under 2 ml of 50 mM Tris/HCl (pH 7.5) with 10% sucrose and 10 mM EDTA, which in turn is overlaid with 2 ml of 50 mM Tris/HCl (pH 7.5), 10 mM EDTA, and centrifuged (160,000 \times g, 17 h, 4°C, Beckman SW55 Ti rotor). Homogenates of 3T3-L1 cells (differentiated for 10 days) are used to establish the gradient. The gradient is divided into four fractions with mean densities of < 1.018 (2 ml), 1.034 (1 ml), 1.055 (1 ml), and 1.098 (1 ml) g/ml, respectively. The major amount of TAG (~60%) is recovered in the $d < 1.018$ g/ml fraction. The pellet (microsomes and cell debris) contains only ~3% of the total amount of TAG recovered from the gradient. Caveolin is present in all fractions and in the pellet.

For gradient II, the samples are adjusted to 40% sucrose, and 1.2 ml is layered under 3 ml of 50 mM Tris/HCl (pH 7.5) with 25% sucrose and 10 mM EDTA, which is in turn overlaid with 1 ml of 50 mM Tris/HCl (pH 7.5) and 10 mM EDTA. After centrifugation (160,000 \times g, 17 h, 4°C Beckman SW55 Ti rotor) the gradient is arbitrarily divided into 5 fractions with mean densities of 1.055 (1.2 ml), 1.099 (1 ml), 1.112 (1 ml), 1.141 (1 ml), and 1.161 (1 ml) g/ml, respectively. Most (75%) of the TAG is present in the top fraction ($d < 1.055$ g/ml). Caveolin is present in the top fraction and in the two bottom fractions.

K.6.1.8.3

Characterization of Lipid Droplets

SDS-PAGE

LD fractions in microcentrifuge tubes are delipidated with 1.5 ml of cold acetone overnight at -20°C followed by centrifugation (14,000 \times g, 30 min, 4°C) and removal of solvent from the protein pellet. The pellet is

further extracted with acetone (20°C) followed by acetone/ether (1/1, by vol.) and ether. Residual solvents are evaporated under nitrogen, and proteins are solubilized in 2 \times Laemmli sample buffer by incubation in a bath sonicator at 65°C for 4–5 h with frequent mixing using a vortex mixer. Additional 2-mercaptoethanol is added to samples before loading onto SDS-PAGE gels. Typically, LD proteins from 28 dishes of adipocytes are loaded onto 30-cm-long SDS-PAGE gels for staining and further identification. Typically, proteins from two dishes of cells are loaded onto gels for transfer to nitrocellulose membranes and immunoblotting. Gels containing greater protein loads are stained for 2 h in 0.25% Coomassie Blue G250 in 10% acetic acid, 50% methanol and then destained in 7% acetic acid, 5% methanol for 4–6 h. To confirm the identification of known LD-associated proteins, nitrocellulose membranes containing LD proteins are probed with antisera raised against the amino terminus of rat perilipin A, mouse adipophilin (Research Diagnostics Inc., NJ), mouse TIP47 (Research Diagnostics), mouse S3-12, and caveolin-1 (Signal Transduction Inc.).

In-Gel Tryptic Digestion of LD-Associated Proteins

Coomassie-stained protein bands are excised from the gels and destained with 45% acetonitrile in 100 mM ammonium bicarbonate. The resulting gel slices are incubated with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride, alkylated by the addition of 50 mM iodoacetamide, and then digested in situ with trypsin (100 ng per band in 50 mM ammonium bicarbonate). The tryptic peptides are extracted using POROS 20 R2 beads (Applied Biosystems) in 0.2% trifluoroacetic acid in 5% formic acid. The extracted peptides are concentrated using C_{18} zip-tips and eluted with 0.1% trifluoroacetic acid in 30% acetonitrile followed by 0.1% trifluoroacetic acid in 75% acetonitrile. The eluates are dried under vacuum using a SpeedVac concentrator.

Mass Spectrometry

The resulting peptides are dissolved in 2–25 μ l of HPLC sample solvents containing water/methanol/acetic acid/trifluoroacetic acid (70/30/0.5/0.01, by vol.). The volume used is proportional to the staining intensity of the given band. Micro-HPLC-MS/MS analysis is conducted on an LCQ electrospray ionization ion trap mass spectrometer coupled with an online MicroPro-HPLC system. Two microliters of tryptic peptide solution is injected into a Magic C18 column (0.2 \times 50 mm, 5 μ m, 200 Å, Michrom BioResources) which had been equilibrated with 70% solvent A (0.5% acetic acid and 0.01% trifluoroacetic

acid in water/methanol (95/5, by vol.) and 30% solvent B (0.5% acetic acid and 0.01% trifluoroacetic acid in methanol:water (95/5, by vol.)). Peptides are separated and eluted from the HPLC column with a linear gradient from 30 to 95% solvent B in 15 min at a flow rate of 2.0 $\mu\text{l}/\text{min}$. The eluted peptides are sprayed directly into the LCQ mass spectrometer (2.8 kV). The LCQ mass spectrometer is operated in a data-dependent mode for measuring the molecular masses of peptides (parent peptides) and collecting MS/MS peptide fragmentation spectra.

Database Search and Protein Identification

The measured molecular masses of parent peptides and their MS/MS data are used to search for information of non-redundant DNA/protein sequence database using the program KNEXUS (Genomic Solutions). The mass error tolerance used in the database search is usually ± 3 Da for the parent ions and ± 0.5 Da for the fragment ions, respectively. Protein identifications are made based on expectation values $< 1 \times 10^{-2}$ or the quality of MS/MS spectra of peptides identified. BLAST searches are performed for hypothetical and unknown proteins.

K.6.1.9

Re-Esterification

PURPOSE AND RATIONALE

In adipose tissue under conditions of enhanced lipolysis, i. e. during fasting or β -adrenergic stimulation, synthesis of TAG from endogenous fatty acids released from the LD during lipolysis is usually not completely repressed. This apparently vicious cycle of the so-called re-esterification is regulated in complex fashion and critically depends on the concentration of the exogenous fatty acids, which in combination with the lipolytically released fatty acids form a common pool for re-esterification. Thus, the supply from/removal by the circulation of fatty acids ultimately determines the rate of re-esterification which thereby ensures rapid and hormone-independent delivery of fatty acids into the circulation in case of immediate demand for nutrients. Previous studies of the regulation of FFA re-esterification *in vitro* have used the balance technique (Vaughan 1962), in which re-esterification is calculated from the difference between glycerol and FFA release (Gilbert et al. 1974, Hammond and Johnston 1987). This method does not distinguish between FFA re-esterification and TAG synthesis. Other studies that measured both processes simultaneously did not provide exogenous FFA in the

incubation medium (Brooks 1982), and so do not accurately mimic the situation *in vivo*, in which FFA substrate may be derived from the plasma, either from hydrolysis of circulating lipoproteins or from albumin-bound FFA.

Whether FFA re-esterification may be regulated independently from TAG synthesis can be addressed by the recently developed dual-isotopic technique for measuring FFA re-esterification in adipose tissue Leibel and Hirsch 1985). This method allows TAG synthesis and re-esterification to be measured independently and simultaneously. Studies using this technique have suggested that the rate of FFA re-esterification is not regulated at the cellular level, but rather by changes in the extracellular environment (Leibel et al. 1989, Edens et al. 1990).

PROCEDURE

Pre-incubation medium consists of Krebs-Henseleit bicarbonate buffer containing 5 g/100 ml BSA and 4.17 mM glucose. Dual isotope incubation medium is a composition identical to pre-incubation medium, except that it also contains 0.5 mM palmitic acid and both [^{14}C]glucose and [^3H]palmitic acid. Palmitic acid is complexed to BSA as described by Leibel and coworkers (1984) with minor modifications. Briefly, a film of mixed unlabeled and [^3H]palmitic acid is dissolved with ethanol and a small (12%) molar excess of NaOH. This solution is heated at 60°C for 30 min with gentle shaking. It is then dried under a stream of N_2 . Pre-warmed (60°C) 10% BSA in distilled, deionized water is added. The 10% BSA is heated and gently shaken until clear. The 10% BSA (now containing 1 mM palmitic acid) is combined with an equal volume of double-strength KHB bicarbonate buffer with glucose to make the incubation medium. The final medium specific activity of [^{14}C]glucose ranges from 0.4 $\mu\text{Ci}/\mu\text{mol}$ to 0.9 $\mu\text{Ci}/\mu\text{mol}$, that of [^3H]palmitate is 2.0 $\mu\text{Ci}/\mu\text{mol}$. The final concentration of glucose in the incubation medium is 5 mM. The gas phase above both pre-incubation and incubation media is 5% $\text{CO}_2/95\%$ O_2 . All incubations are carried out 2 h at a shaking speed of 80 cycles/min and temperature of 37°C. Zero time samples are run and all other values are corrected accordingly. The isolated rat adipocytes are suspended at a 50% concentration and aliquoted (200 μl) into 2 ml of incubation medium. Incubations are done in 20-ml polypropylene, screw-top vials at 37°C, with shaking at 80 cycles/min. Incubations in medium containing either no added FFA or 0.5 mM palmitic acid are done in the basal state (no additions) and either with adenosine deaminase to prevent inhibition of lipoly-

sis or with isoproterenol (1 μM) to stimulate lipolysis. At the end of the incubations (2 h), the adipocytes and medium are poured into iced Falcon tubes containing PE90 catheters. The tubes are spun gently for 1–2 min at 0°C to float the adipocytes. Incubation medium is withdrawn from beneath the adipocytes through the catheter and frozen for subsequent glycerol assay by the method of Laurell and Tibbling (1966). The cells remaining in the tube are extracted by the method of Dole and Meinertz (1960). The extracted lipids are subjected to TLC to isolate TAG. The TAG is recovered and counted simultaneously for both ^3H and ^{14}C . The incorporation of [^{14}C]glucose into TAG calculate the rate of re-esterification as described (Leibel and Hirsch, 1985).

EVALUATION

Under these experimental conditions, ^{14}C is not incorporated into the fatty acid moiety of AG and ^3H is not incorporated into the glycerol moiety. FFA re-esterification can be measured because both unlabeled FFA arising from lipolysis and medium-derived [^3H]palmitate are esterified in newly synthesized [^{14}C]AG. The degree of dilution of [^3H]palmitate by unlabeled, endogenous FFA in [^{14}C]TAG is an index of the rate of re-esterification. Re-esterification is calculated as the difference between the total theoretical moles of FFA in TAG ($\mu\text{mol}[\text{C}^{14}]\text{TAG} \times 3$) minus the actual total μmoles of [^3H]palmitate esterified into TAG. Therefore, FA re-esterification, or the esterification of unlabeled FFA in TAG equals $[(3 \times \mu\text{mol}[\text{C}^{14}]\text{TAG}) - \mu\text{mol}[\text{H}^3]\text{TAG}]$, where μmoles of [^3H]TAG represents the amount of [^3H]palmitate esterified into [^{14}C]TAG. The data are expressed as μmol FFA re-esterified per μmol newly synthesized TAG $(3 \times \mu\text{mol}[\text{C}^{14}]\text{TAG} - \mu\text{mol}[\text{H}^3]\text{TAG} / \mu\text{mol}[\text{C}^{14}]\text{TAG})$, a value that theoretically can vary between zero (no re-esterification) and three (all newly esterified fatty acids derived from lipolysis). Adipocyte size is measured in aliquots of isolated adipocytes by microscopy. The lipid content of aliquots of isolated adipocytes is determined gravimetrically in Dole extracts. Adipocyte concentration in the incubation mixture is calculated by dividing lipid content by cell size (assumed to be 100% lipid).

K.6.1.10

Cellular Lipolysis

GENERAL CONSIDERATIONS

Deregulation of lipid metabolism has long been recognized as essential in the development of obesity and

the metabolic syndrome. White adipose tissue (WAT) lipolysis plays a pivotal role in controlling the quantity of TAG stored in fat depots and in determining plasma free fatty acid levels (Frayn 2002). Hence, targeting critical steps of this catabolic process constitutes one of the strategies to combat obesity and the metabolic syndrome (Nisoli and Carruba 2004).

Activators of lipolysis represent a pharmacological interest only if the same molecule or another compound stimulates oxidation of fatty acid and energy expenditure. The lipolytic and thermogenic β_3 -adrenergic agonists highly efficient in rodents to decrease fat mass and insulin resistance have so far not been effective in humans. There are several potential explanations. Unlike rodents, adult humans have very little brown adipose tissue, the tissue specialized in thermogenesis in rodents, which possesses a lot of β_3 -adrenoceptors. The receptors are expressed at low levels in human white adipocytes, again a major difference with rodents. Finally, the first generation of β_3 -adrenergic agonists may have low efficacies and potencies for the human β_3 -adrenergic receptor. Nevertheless, the approach is interesting conceptually. Ideally, a strategy could be to stimulate lipolysis and use of the fatty acids by newly formed brown fat cells (Tiraby and Langin 2003, Langin 2006). Concomitant stimulation of fat oxidation in skeletal muscle is an alternative strategy. The recently characterized lipolytic receptors (e.g., natriuretic peptide and pituitary adenylate cyclase-activating polypeptide receptors) and pathways (e.g., residual lipolytic effect of catecholamines in mice with no β -adrenoceptors) may constitute novel drug targets.

Since the launching of nicotinic acid (niacin) as a lipid-lowering drug a long time ago, suppression of lipolysis to decrease free fatty acid level has attracted much interest (Karpe and Frayn 2004). However, most known antilipolytic receptors are expressed in several organs raising the risk for side-effects. The recent cloning of the receptor for nicotinic acid which is mainly expressed in WAT has undoubtedly led to important screening efforts for the identification of agonists with less side-effects than niacin and its long-lasting form, acipimox (Tunaru et al. 2003, Wise et al. 2003). Inhibition of HSL is also attractive as the enzyme has little homology with other mammalian lipases and show a rather limited tissue distribution. Indeed, several series of agonists have been synthesized (Slee et al. 2003, Ebdrup et al. 2004) with an apparent high specificity for some of them (Langin et al. 2005). The effect of chronic treatment in rodent models of obesity and dyslipidaemia is now awaited.

PURPOSE AND RATIONALE

The inhibition of lipolysis induced by starvation or β -adrenergic stimulation in response to insulin is of exquisite sensitivity ($IC_{50} < 0.1$ nM) and responsiveness (lipolysis rate less than 5% of that in the basal state) toward the hormone. This reflects the enormous physiological importance of a tight regulation of fatty acid release from adipose tissue to ensure both adequate nutrient supply and prevention from the detrimental so-called lipotoxic effects of FFA in the circulation. The complex molecular mechanism of the signal transduction in the adipocytes from the insulin receptor downstream to the lipolytic end effector systems, i. e. the TAG-degrading lipases (see K.6.1.13) has been characterized in detail (for a review see Müller and Petry 2005). It encompasses the translocation of lipases (HSL) and LD surface proteins (perilipins) from the cytosol to the LD and *vice versa*, respectively, which is stimulated in the fastened/ β -adrenergic state and blocked in response to insulin. These translocation processes are regulated by (PKA-dependent) phosphorylation of both the lipases and LD surface proteins and presumably additional regulatory proteins. These phosphorylations are down-regulated by insulin through stimulation of cAMP degradation by phosphodiesterase 3B which is linked directly to distal elements of the insulin signaling cascade in adipocytes. Basal and stimulated lipolysis as well as its inhibition is monitored *in vitro* with cultured and primary rodent and human adipocytes and can be used to study insulin-like effects on the negative regulation (i. e. activation of the insulin signaling) or direct inhibition of lipolysis (i. e. of the lipases involved) by compounds/drug candidates. Several procedures are available based on the determination of glycerol and fatty acids released from unlabeled, fluorescently labeled or radiolabeled TAG.

K.6.1.10.1**Method Based on Isolated Fat Pads**

Gonadal fat pads are removed from male rats and washed several times with PBS. Tissue pieces (~20 mg) are incubated in DMEM containing 2% fatty acid-free BSA with or without 10 μ M isoproterenol at 37°C. Portions are collected after 30, 60, 120, 180 and 240 min and analyzed for the FFA and glycerol content by using commercial kits (Wako Chemicals).

K.6.1.10.2**Method Based on the Release of Fluorescent Fatty Acids from Isolated Adipocytes****PROCEDURE**

This method which avoids the use of radioactivity but nevertheless enables the visualization and quantitative evaluation of the released fatty acids has been introduced by Müller and coworkers (2003). For fluorescent labeling of adipocyte TAG, 250- μ l portions of adipocyte suspension in 1.5-ml plastic cups are incubated (15 min, 37°C) with 200 μ l of KRH containing 1% BSA in the presence of 0.55 nM insulin prior to addition of 50 μ l of NBD-FA (500 μ M, prepared daily by dissolving 39.8 mg NBD-FA in 1 ml ethanol under moderate heating and subsequent 1:200 dilution with KRH containing 1% BSA) and further incubation (1 h, 37°C) under mild shaking (thermomixer 5436, setting 10, Eppendorf, Hamburg, Germany). For removal of free NBD-FA left in the incubation medium, the adipocyte suspensions are combined in 50-ml plastic vials and centrifuged (500 \times g, 1 min, swing-out rotor). The infranatant below the cell surface layer is aspirated. The adipocytes are suspended in 30 ml of medium L (Medium 199 containing 25 mM HEPES, 4 mM L-glutamine, 5.5 mM glucose, 3% BSA). After two further cycles of centrifugation and resuspension, the washed adipocytes are finally adjusted to 2.5×10^6 cells/ml in medium L. For treatment with compounds/drug candidates directly inhibiting relevant lipases which may bind to proteins, the adipocytes are washed with medium L lacking BSA and then incubated (15 min, 37°C) with the inhibitors in the same medium. For initiation of lipolysis, 400- μ l portions of adipocyte suspension are supplemented with 500 μ l of medium L containing 2 or 4% BSA (in case of pre-incubation with inhibitors) and 5.5 mM glucose and incubated (37°C) for 30–180 min in the presence or absence of combinations of isoproterenol and ADA in plastic scintillation vials (total vol. 1 ml) under mild shaking in a water bath. 150- μ l Portions of the suspension are transferred into pre-cooled Eppendorf cups containing 750 μ l chloroform/heptane/methanol (3/3/2 by vol.), mixed vigorously and centrifuged (12,000 \times g, 1 min). The lower organic phase is removed, dried (SpeedVac) and then suspended in 20 μ l of THF. 5- to 10- μ l portions are analyzed by TLC using Si-60 F₂₅₄ plates (Merck, Darmstadt, Germany) and diethylether/petrolether/acetic acid (78/22/1, by vol.) as solvent system.

EVALUATION

Quantitative evaluation of the amount of NBD-FA is performed by fluorescence imaging (Storm 860, Molecular Dynamics, Germany) using imaging software (Molecular Dynamics, ImageQuant). Alternatively, 20- μ l aliquots of the lower phase are supplemented with 325 μ l of methanol/chloroform/heptane (10/9/7, by vol.) and 1.05 ml of 0.1 M potassium carbonate, 0.1 M boric acid (pH 10.5), vortexed and then centrifuged (12,000 \times g, 1 min). 150- μ l Portions of the upper phase are removed and measured for fluorescence (automatic fluorometric analyzer, Cobas Bio, Roche Diagnostics, Germany).

K.6.1.10.3**Method Based on the Release of Glycerol from Isolated Adipocytes****PROCEDURE**

The determination of glycerol released from unlabeled adipocytes is the most common and convenient method and can be performed according to published procedures (Petry et al. 2005) with the following modifications. 300- μ l aliquots of the incubation mixtures are rapidly transferred into 1.5-ml Eppendorf cups precooled to 4°C and containing 300 μ l of 10% (w/v) HClO₄. After incubation (1 h, 4°C), the precipitates are removed by centrifugation (12,000 \times g, 2 min). The infranatants recovered are neutralized with 20% KOH, followed by addition of 60 μ l of 1 M Tris/HCl (pH 7.4). 150- μ l samples are removed for glycerol determination, frozen in liquid N₂ and stored at -20°C. Glycerol is measured fluorometrically by a modified version of the enzymatic method (Wieland 1974). The reaction mixture contains 0.1 M Tris/HCl (pH 9.3), 0.9 mM ATP, 1.25 mM NAD, 0.1 M MgCl₂, 0.25% (v/v) hydrazine hydrate, 5–50 μ l sample or glycerol standard, and a mixture of glycerol kinase (0.2 U/ml final conc.) and glycerol 3-phosphate dehydrogenase (0.7 U/ml final conc.) in a total volume of 240 μ l. Fluorescence is determined using an automatic fluorometric analyzer.

K.6.1.10.4**Method Based on the Release of [³H]Oleic Acid from Isolated Adipocytes****PROCEDURE**

Adipocytes are washed with KRPH buffer (128 mM NaCl, 4.7 mM KCl, 1 mM MgCl₂, 1.25 mM CaCl₂, 25 mM sodium phosphate, 1 mM sodium pyruvate, 5.5 mM glucose, 50 mM HEPES/KOH, pH 7.4) and then incubated (2 h, 37°C) in fresh KRPH contain-

ing [³H]oleic acid (50 μ Ci per 2 \times 10⁶ cells and ml) and 1% BSA in the presence of 0.55 nM human insulin. The adipocytes are washed with KRPH (10°C) containing 5.5 mM glucose and 2% BSA or lacking BSA (for subsequent treatment with direct lipase inhibitors for 15 min at 37°C) three times by centrifugation and then incubated (2 h, 37°C) under mild shaking in KRPH containing 5.5 mM glucose, 2% BSA and 400 μ M phloretin (for reduction of fatty acid uptake by 85%) at 1 \times 10⁶ cells/ml in the presence of lipolytic stimuli. Thereafter, 150- μ l portions of the adipocyte suspension are transferred into narrow centrifugation tubes (Beckman) and then overlaid with 100 μ l of dinonylphthalate (Merck, Darmstadt, Germany). After centrifugation (2,000 \times g, 30 sec, 25°C), the tubes are cut through the oil layer and the lower parts of the tubes containing the incubation medium transferred into 5-ml scintillation vials and mixed with 3 ml of chloroform/heptane (1/1, by vol.) containing 2% methanol. After centrifugation (2,000 \times g, 5 min), the upper aqueous phase is removed by suction. Following addition of 5 ml of liquid scintillation cocktail to the lower organic phase, radioactivity is measured.

EVALUATION

Control values of the 0-min incubation with the corresponding lipolytic stimulus are subtracted in each case to correct for [³H]oleic acid left from the labeling period and accounting for 2–7% of the total radioactivity recovered with the medium. TLC and phosphorimaging analysis revealed that both under basal and lipolytic conditions, [³H]oleic acid accounts for more than 70% of the total radioactivity measured, radiolabeled degradation products (recovered mainly with the aqueous phase) for 15–20% and TAG for up to 6%.

K.6.1.10.5**Method Based on the Release of Unlabeled Fatty Acids from Isolated Adipocytes****PURPOSE AND RATIONALE**

Studying lipolysis with human adipocytes and adipose tissues is hampered by methodological problems. The amount of human adipose tissue that is available is usually limited and the rate of lipid mobilization from human adipocytes is low compared to that of rat adipocytes. Both chemical and enzymatic methods for determination of FFA in human blood and serum have been developed (e. g. Matsubara et al. 1983, Shimizu et al. 1980, Duncombe and Rising 1973, Miles et al. 1983, Okabe et al. 1980). However, incomplete recovery of FFA from plasma (Duncombe and Rising 1973)

and interference of BSA with the FFA assays (Matsubara et al. 1983, Shimizu et al. 1980, Okabe et al. 1980) have been reported, which results in an underestimation of FFA values in serum and plasma samples. To overcome this, the extraction method has been modified (Duncombe and Rising 1973), BSA has been included in the standard curve (Miles et al. 1983) or p-toluenesulfonic acid has been used (Matsubara et al. 1983).

These methods are unsuited for FFA determination for human *in vitro* studies since the detection limit is too high using small amounts of human adipose cells with usually low lipolytic activity. Näslund and coworkers (1989) have established a chemiluminometric method for the determination of low levels of FFA based on H₂O₂ determination in a peroxidase luminescence reaction. Since albumin (which must be added to the incubation medium as FFA carrier) interferes with this assay resulting in non-linear FFA standard curves, Näslund and coworkers (1993) modified the chemiluminometric assay for compatibility with biological samples containing albumin.

PROCEDURE

A semiautomatic luminometric method for determination of small amounts of FFA released from human adipocytes *in vitro* has been introduced by Näslund and coworkers (1993). BSA is used as acceptor of FFA in the incubation medium of isolated fat cells. The assay involves pretreatment with the detergent SDS to liberate the FFA from the BSA before activation by acyl-CoA synthetase (ACS). This is followed by oxidation of the resulting thioesters by acyl-CoA oxidase (ACO). The H₂O₂ formed is subsequently measured in a horseradish peroxidase (HRP)-catalyzed luminol reaction. The assay is linear in the interval of 0.01–1 nmol in the cuvette corresponding to 2–200 μ moles in the sample, and 25 samples can be automatically assayed in the luminometer within 75 min. FFA release can easily be studied in a small incubation volume (200 μ l) of very diluted (10⁴ cells/ml) human adipocyte suspensions. Samples (25 μ l) containing 0.25% BSA from incubates of adipose tissue cells do not interfere with the standard curve. This method (Näslund et al. 1993) is sensitive, simple and inexpensive luminometric assay for FFA release. It is many 100-fold more sensitive than standard spectrophotometric methods and can be used for serial studies of lipid metabolism (lipolysis, FFA re-esterification) in small amounts of human adipose tissue or for investigations of FFA release from very diluted human adipose cell samples.

K.6.1.11

Cell-Free Lipolysis

PURPOSE AND RATIONALE

For analysis of effects of compounds/drug candidates on the translocation to the LD and direct activation of HSL including the increase in accessibility of the TAG of LD for degradation by HSL (or other/additional lipases), these individual steps of lipolysis can be reconstituted in a cell-free system consisting of purified LD and crude HSL (and other lipases) prepared from primary rat adipocytes.

PROCEDURE

Preparation of LD from Adipocytes

LD are prepared according to Egan and coworkers (1992) and adaptations from Wolins and coworkers (2005) with the following modifications: After the incubation, the adipocytes are washed two times with KHH by flotation and removal of the infranatant by suction and then homogenized in 25 mM Tris/HCl (pH 7.4), 250 mM sucrose, 5 mM NaF, 10 mM NaPP_i, 1 mM Na₃VO₄, 1 mM EDTA, 20 μ g/ml leupeptin, 1 mM benzamidine, 0.5 mM PMSF at 15°C. After centrifugation (1,000 \times g, 5 min, 15°C), 1 ml of the supernatant is combined with 1.5 ml of 65% sucrose (w/v) and poured into a 5-ml centrifuge tube. 1.5 ml of 10% sucrose (w/v) is then layered on top of the sucrose cushion. The tube is filled to capacity with buffer A. The gradient is centrifuged (172,000 \times g, 60 min, 15°C) and then allowed to coast to rest. The floating fraction of LD is visualized as the upper white layer of the gradient, and is isolated by suction with a syringe (0.8 ml). After one washing cycle with buffer A, the LD are suspended in an equal volume of 4-fold sample buffer containing 10% SDS by incubation (10 min, 65°C) and treatment in a bath sonicator (10 min, 25°C, max. power) for extraction of proteins. After centrifugation (10,000 \times g, 5 min, 25°C), proteins contained in the infranatant are separated by SDS-PAGE. Alternatively, the gradient is fractionated from bottom to top by suction with a syringe (0.8 ml each). Proteins of fractions 1–5 are precipitated with 5% TCA (15 min, 2°C, 15,000 \times g, 10 min), washed three times with ice-cold acetone, dried and finally suspended in sample buffer. Fraction 6 is extracted for protein (see above). All samples are analysed by SDS-PAGE and immunoblotting.

Cell-Free System

Adipocytes labeled with NBD-FA (see K.6.1.2.2) are washed with KRH containing 0.5% BSA, suspended

in the same medium at 3.5×10^5 cells/ml and incubated (5 min, 37°C) in plastic scintillation vials under mild shaking in a water bath in the presence or absence of isoproterenol, insulin or compound/drug candidate. Adipocytes (3.5×10^6 cells) are washed twice with BSA-free KRH by flotation ($500 \times g$, 1 min) and then suspended in 2.5 ml of homogenization medium (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 2 mM DTT, 50 mM glycerol-3-phosphate, 10 mM sodium pyrophosphate, 25 mM NaF, 1 μ M microcystin, 2 μ M cantharidin, 1 mM benzamidine, 5 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml antipain, 25 μ g/ml soy bean trypsin inhibitor, 5 μ g/ml PKA inhibitor). The mixture is vortexed, homogenized in a teflon-in-glass homogenizer (10 strokes using a rotating teflon pestle) at 15°C, transferred into plastic vials (under rinsing of the homogenization vessel with homogenization medium) and then centrifuged ($12,000 \times g$, 30 min, 4°C, swing-out rotor). The infranatant (cytosol) is separated from the (fluorescent) TAG-containing LD by aspiration with a syringe. To eliminate entrapped unbroken cells, the LD are resuspended in 8 ml of homogenization medium (15°C) containing 120 mM sucrose and 140 mM KCl, vortexed vigorously, and subsequently subjected to centrifugation ($200,000 \times g$, 1 h). The infranatant (washing medium) is removed by suction. For initiation of NBD-FA release, 1 ml of ice-cold homogenization medium containing 2% BSA is added to the plastic vial while the LD adhering to the vessel wall are removed by repeated pipetting. A teflon-coated magnetic stirring disc is placed into the vial. The mixture is warmed up to 30°C and incubated under stirring (250 rpm) for 1 to 4 h. The digestion is terminated by addition of 2 ml of chloroform/methanol (3/1; by vol.). The extracts are dried (SpeedVac), suspended in 50 μ l of THF and then analyzed by TLC as described below.

Cell-free lipolysis is performed in glass tubes as described by Okuda and coworkers (1994) and Morimoto and coworkers (2000, 2001) with the following modifications: 40 μ l of packed NBD-FA-labeled LD are incubated (90 min, 30°C, constant shaking) with 80 μ l of a solution enriched for HSL (100–200 μ g protein) and 120 μ l of buffer C containing 2% BSA, 2 mM DTT with or without 4.2 mg gum arabic. For incubation of the cell-free lipolysis system, compounds are added directly from the 10-mM stock solutions in DMSO to the reaction mixture containing the LD without prior dilution. The final DMSO concentration during all incubations is kept constant at 0.1%. Portions of the lipolysis reaction are terminated by extraction of the total incubation mixture with 720 μ l

of chloroform/methanol/0.1N HCl (3/1/1, by vol.). After vortexing and centrifugation ($2,000 \times g$, 2 min) of the mixture, the organic phase is collected, dried under a stream of N_2 and resuspended in 40 μ l of tetrahydrofuran. 10- μ l portions are analyzed by TLC (Silica Gel G plates, Merck Darmstadt, Germany; diethylether/petrolether/acetic acid, 78/22/1 by vol.). NBD-FA released from the total NBD-FA-labeled AG is quantitatively evaluated by phosphorimaging (e.g. Storm 860, Molecular Dynamics, Germany) using imaging software (ImageQuant, Molecular Dynamics), as described previously (Müller et al. 2003).

EVALUATION

A blank value of a control reaction terminated immediately after addition of HSL-solution to the LD is subtracted in each case. Other portions of the lipolysis reaction are supplemented with 760 μ l of buffer C containing protease inhibitors and centrifuged through sucrose cushions for the recovery of LD which are then analyzed for protein composition, in particular for the amount of HSL (HSL translocation, see K.6.1.12).

K.6.1.12

Translocation of Hormone-Sensitive Lipase (HSL)

PURPOSE AND RATIONALE

The molecular mechanism of the regulation of lipolysis in adipocytes by starvation, β -adrenergic stimuli and insulin as well as compounds/drugs (e.g. nicotinic acid) relies on the movement of HSL from the cytosol, its predominant location in the basal state, to the LD (Egan et al. 1992, Brasaemle et al. 2000, Clifford 2000, for reviews see Holm 1999 and 2003, Londos 2005, Müller et al. 2005). Candidate components involved in this HSL translocation initiated by β -adrenergic stimuli and inhibited by insulin and lipid-lowering drugs are elements of the cytoskeleton and LD-associated proteins, such as perilipin. Interestingly, perilipin has been observed to undergo reverse translocation from the LD to the cytosol upon challenge of adipocytes with insulin. The relative contribution of HSL and perilipin translocation to lipolysis stimulation seems, however, to depend on size, age and origin of the adipocytes. HSL translocation blocked by insulin or compounds can be easily monitored by immunoblotting of isolated LD for HSL.

PROCEDURE

For the identification of HSL associated with LD in the induced compared to the basal state of the adipocytes, LD are prepared, purified, suspended in 20% SDS,

vortexed vigorously, then incubated (10 min, 95°C) and finally centrifuged (12,000×g, 5 min). Solubilized LD-associated proteins recovered from the infranatant by suction are analyzed by SDS-PAGE (7.5% separating gel). The proteins are then transferred onto polyvinylidene difluoride (PVDF) membranes for 4 h at 200 mA using 20 mM 3-(cyclohexylamino)-1-propanesulphonic acid (pH 11.0) in 10% methanol. Transfer efficiency is evaluated by Amido black staining in control lanes. The membrane is washed with PBS containing 0.1% Tween-20, then incubated (1 h, 25°C) with PBS containing 0.5 M NaCl and 0.75% BSA and subsequently incubated (18 h, 4°C) in the same buffer with anti-HSL antibody (1:1,000 dilution, 0.5–2 µg/ml). This antibody was raised in white New Zealand rabbits against recombinant full-length His-tagged human HSL. This 84-kD polypeptide was encoded by a pET-derived expression vector under the control of the T7 promoter, expressed in *E.coli*, then partially purified from inclusion bodies by SDS-PAGE and subsequent elution from the gel band and finally homogenized in PBS prior to emulsification in Ribi Adjuvant System (RIBI Immunochem Research Inc., USA) and subsequent intraperitoneal injection into rabbits. The antibody was purified by chromatography on protein A-Sepharose. Subsequently, the blotting membrane is washed two times with PBS containing 0.1% SDS, 0.2% Nonidet P-40, and 0.25% sodium deoxycholate and then once with PBS. Thereafter the filters are incubated (2 h, 25°C) with horseradish peroxidase conjugated anti-rabbit-IgG (1:5,000 dilution) in PBS containing 0.75% BSA.

EVALUATION

Following three washing cycles (see K.6.1.4.3.1, K.6.3.1.3.2.1 and K.6.3.4.3), the amount of HSL is determined by ECL Western blotting detection reagents (Amersham-Pharmacia, Germany) according to the manufacturer's instructions. Quantitative evaluation is performed by luminometric scanning (Roche Diagnostics, Germany) using ImageQuant software.

K.6.1.12.1

Protein Composition of LD

PURPOSE AND RATIONALE

Stimulation as well as inhibition of lipolysis in adipocytes by hormones, nutritional signals or compounds/drug candidates exerts marked effects on the protein composition of LD favoring TAG/LD degradation or TAG/LD synthesis, respectively. These alterations can be monitored by modern proteomic anal-

ysis as has already been performed by Brasaemle and coworkers (2004) for 3T3-L1 adipocytes, Liu and coworkers (2004) for CHO fibroblasts, Fujimoto and coworkers (2004) for cultured human HuH7 hepatoma cells, Umlauf and coworkers (2004) for cultured human A431 epithelial cells and Wu and coworkers (2000) for mouse mammary glands. The unexpected identification of caveolin as LD-associated protein in adipocytes (for a review see Liu et al. 2002) demonstrates the power of modern proteomic analysis. These data may be useful to characterize and differentiate the molecular mechanisms of compounds which interfere with the lipid metabolism of physiologically relevant cells, such as adipocytes, at unknown site(s).

PROCEDURE

For proteomic analysis of adipocyte LD, six days after the initiation of differentiation, 40 dishes of 3T3-L1 adipocytes are incubated either under lipolytically stimulated conditions (e. g. 10 µM isoproterenol and 0.5 mM IBMX for 15 min at 37°C) or without the additions for basal conditions and subsequently in the absence or presence of anti-lipolytic stimuli (e. g. insulin or compounds/drug candidates for 2 h) before harvest. Culture medium is removed, and cells are rinsed twice with ice-cold PBS before scraping of the cells into PBS using typical cell scrapers. Cells from sets of 10 dishes of cells are pooled into 15-ml conical screw-capped tubes (Falcon) and centrifuged (500×g, 5 min). Cell pellets are resuspended in a hypotonic medium containing 10 mM Tris (pH 7.4), 1 mM EDTA, 10 mM sodium fluoride, 20 µg/ml leupeptin, 1 mM benzamidine, and 100 µM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride by pipetting and incubated on ice for 10 min before homogenization by 10 strokes in a Teflon-glass homogenizer. Cell lysates are centrifuged (26,000×g, 30 min, 4°C, SW41 Ti-rotor Beckman), and the rotor is allowed to coast to a stop. The floating LD layers are harvested using a tube slicer, and the harvested fractions are adjusted to 25% sucrose and 100 mM sodium carbonate (pH 11.5) using a 60% sucrose stock solution and a 1 M sodium carbonate stock solution with protease inhibitors followed by gentle mixing by pipetting. The density-adjusted fractions (~4 ml) are layered into centrifuge tubes containing 1-ml cushions of 60% sucrose and then overlaid with ~5 ml of 100 mM sodium carbonate (pH 11.5) with protease inhibitors followed by ~3.5 ml of the hypotonic lysis medium with protease inhibitors. Tubes are centrifuged (26,000×g, 30 min, 4°C, SW41 Ti-rotor Beckman) and the rotor is allowed to coast to a stop.

Floating LD are harvested using a tube slicer into 1.5-ml microcentrifuge tubes. Residual carbonate solution is removed by centrifuging tubes ($14,000 \times g$, 20 min, 4°C , Eppendorf microcentrifuge). The infranant is removed with an 18-gauge needle from below the floating LD fraction, and the LD fraction is rinsed once with hypotonic lysis solution containing protease inhibitors.

EVALUATION

Typically, LD are isolated from 8 separate sets of 40 dishes of adipocytes grown and differentiated at different times. 4 sets of dishes are used for basal conditions, and 4 sets of dishes are used for lipolytically stimulated conditions. Proteins from LD preparations from 3 sets of 28 dishes of basal adipocytes are separated in 3 lanes of a single SDS-PAGE gel. Coomassie-stained bands are compared and found to be equivalent for all lanes. Bands from one lane are excised for analysis by mass spectrometry. Proteins from LD preparations from 3 sets of 28 dishes of lipolytically stimulated adipocytes are separated in 3 lanes of a single SDS-PAGE gel and compared with a single lane containing proteins from 28 dishes of basal adipocytes on the same gel. Coomassie-stained bands are compared and found to vary slightly between the three preparations from the lipolytically stimulated adipocytes and significantly between basal and stimulated preparations. Bands are excised for all stained proteins from two lanes of stimulated preparations and analyzed by mass spectrometry.

The LD of adipocytes are unique among LD of many types of cells with respect to size, the perilipin-enriched protein composition, and the dynamic rearrangements in structure that occur in response to stimulation of the β -adrenergic signaling pathway. Although perilipin A is the most abundant protein associated with the large LD of basal adipocytes, when lipolysis is stimulated, these LD fragment into myriad micro-LD. Interestingly, the total mass of perilipin does not increase in proportion to the greatly increased surface area of the micro-LD. Some of the excess surface becomes coated with other PAT family members, adipophilin, TIP47, and S3-12 (Wolins et al. 2005). Additionally, the association of numerous unrelated proteins with the LD increases.

Adipophilin was found by proteomic analysis of proteins on LD isolated from lipolytically stimulated 3T3-L1 adipocytes but not basal adipocytes. This finding was confirmed by immunofluorescence microscopy experiments. The appearance of adipophilin on the LD of lipolytically stimulated adipocytes may

be a consequence of increased availability of binding sites on micro-LD to stabilize nascent adipophilin. The results strongly suggest that adipophilin plays a role in lipolysis. The identification of caveolin-1 on LD isolated from lipolytically stimulated but not basal adipocytes is consistent with previous observations (Cohen et al. 2004). Caveolin-1, perilipin, and the catalytic subunit of PKA can be co-immunoprecipitated from lysates of adipocytes preincubated with β -adrenergic receptor agonists but not from basal adipocytes (Cohen et al. 2004). Thus, the three proteins form a complex in stimulated cells with caveolin-1 thereby bridging between perilipin and the catalytic subunit of PKA to facilitate the phosphorylation of perilipin. The identifications of lipid metabolic enzymes on the LD of adipocytes and several other types of cells shows that these structures are not passive repositories for neutral TAG but, instead, comprise a dynamic pool of lipids.

The identifications of several proteins in the adipocyte LD preparations carry implications regarding the immediate subcellular neighborhood of LD. The intermediate filament protein, vimentin, is identified in the basal and stimulated state. Previous studies show that fibrous vimentin intermediate filaments collapse into a cage structure around developing LD in differentiating adipocytes (Franke et al. 1987). The disruption of vimentin cage formation halts LD formation (Lieber et al. 1996). Identification of the Eps-15 homology domain protein EHD2, a protein that binds to actin filaments in differentiated 3T3-L1 adipocytes (Guilherme et al. 2004), provides additional evidence of a close link between LD and the cytoskeleton. The identification of calnexin in basal and lipolytically stimulated LD preparations, as confirmed by immunofluorescence microscopy, suggests that segments of endoplasmic reticulum come into close contact with LD. Finally, several mitochondrial proteins can be identified in both preparations, including a subunit of ATP synthase, prohibitin, and pyruvate carboxylase, indicating that mitochondria are closely associated with and difficult to separate from LD. Several studies have illustrated the tight packing of mitochondria around LD in adipocytes (Cohen et al. 2004, Blanchette-Mackie et al. 1995).

In conclusion, surface proteins on adipocyte LD include enzymes involved in many aspects of lipid metabolism, as found on LD in other types of cells. However, unlike other types of LD, perilipins are a major protein component that controls lipid traffic and the association of adipophilin (Brasaemle et al. 1997, Tansey et al. 2001, 2003), HSL (Sztalryd et al. 2003),

CGI-58 (Yamaguchi et al. 2004, Subramanian et al. 2004), and likely of other proteins with the LD. The stimulation of lipolysis is accompanied by the fragmentation of large LD into numerous dispersed micro-LD accompanied by major changes in the overall protein composition. Although most of the changes appear to affect the relative mass of various proteins that also associate with basal LD, adipophilin and caveolin-1 selectively associate with LD in stimulated cells. It is expected that this type of analysis will be useful for the future characterization including elucidation of the mode of action of compounds/candidate drugs which affect lipid metabolism by either stimulating or inhibiting TAG synthesis or degradation and in consequence LD biogenesis in adipose as well as in non-adipose tissues.

K.6.1.12.2

Interaction of HSL and Perilipin

PURPOSE AND RATIONALE

Recently, the differential effects of blocking perilipin phosphorylation on HSL translocation and lipolysis have been dissected (Miyoshi et al. 2006). Employing adenoviral and transgenic expression of a perilipin construct deficient in all 6 PKA sites (Peri A $\Delta 1-6$), PKA-dependent phosphorylation of perilipin has been demonstrated not to be required for HSL translocation to the LD, but is essential for the lipolytic actions of LD-associated HSL in adipocytes. Crosslinking and immunoprecipitation studies suggest that PKA-dependent phosphorylation of perilipin promotes close-range interaction with LD-associated HSL. It is conceivable that compounds/drug candidates promoting or abrogating TAG synthesis/LD biogenesis or their degradation modify the HSL-perilipin in negative or positive fashion, which can be monitored with the following assay systems.

PROCEDURE

Construction of Perilipin KO Mice Expressing the Perilipin Mutant Transgene Lacking the 6 PKA Consensus Phosphorylation Sites in BAT

A targeted disruption of the perilipin gene is performed by replacing exon 3 with a neomycin cassette, thereby disrupting coding of all known perilipin mRNAs. The perilipin KO mice are viable and exhibit predicted reductions in adipocyte size and adipose mass (Martinez-Botas et al. 2000). Perilipin KO mice are backcrossed for 10 generations to C57BL/6 mice. For generation of mice expressing a peri $\Delta 1-6$ transgene in adipose tissue, a mouse peri $\Delta 1-6$ cDNA, FLAG-

tagged at the carboxy-terminal using PCR is generated and then ligated into a SmaI site of pBluescript SK vector containing the aP2 enhancer/promoter region, the SV40 small tumor antigen splice site and polyadenylation signal sequence. A fragment containing the entire aP2-perilipin A-FLAG transgene is microinjected into fertilized eggs of C57BL/6 mice using well-described procedures (Brinster et al. 1981). Transgenic mice expressing the peri $\Delta 1-6$ transgene almost exclusively in BAT are obtained. These mice are fully viable, exhibit no apparent adipose phenotype and are mated with backcrossed perilipin KO mice to generate perilipin A $\Delta 1-6$ mice (Miyoshi et al. 2006).

HSL-Perilipin Crosslinking and Coimmunoprecipitation

Differentiated adipocytes expressing GFP and wild-type or mutant (e. g. deletion of the 6 PKA consensus phosphorylation sites) FLAG-tagged perilipin A are incubated in differentiation medium containing palmitic and oleic acid (240 μ M each) for 48 h. After overnight serum depletion, adipocytes are treated for 15 min with 200 nM phenylisopropyl adenosine (PIA, basal state) or 20 μ M forskolin *plus* 0.5 mM IBMX (stimulated state), washed with PBS and then maintained for 10 min in hypotonic lysis buffer (20 mM HEPES/KOH, pH 7.5, 2 mM EDTA, 50 mM sodium fluoride, 100 μ M sodium orthovanadate, protease inhibitor cocktail) and either PIA or forskolin *plus* IBMX. Cells are then harvested and homogenized (10 strokes with a teflon-in-glass homogenizer on ice). 3,3'-Dithiobis sulfosuccinimidylpropionate (DTSSP), a cross-linker with 12A spacer arm length (Pierce, Rockford) is added to the homogenates and incubated with rotation at 22°C for 30 min. The crosslinking reaction is terminated by addition of Tris/HCl (50 mM final). After addition of Triton X-100 (2% final) and NaCl (75 mM final), samples are drawn through a 23-gauge needle and then centrifuged (10,000 \times g, 20 min, 4°C) to separate the LD (upper layer), infranatant (lower layer) and a membrane pellet. The LD are isolated with a tube cutter and placed in a fresh tube. The infranatant is subjected to multiple rounds of centrifugation and removal of the residual LD, until almost no LD are visible. The pooled LD fractions are washed with 1 ml of fresh lysis buffer, collected after centrifugation and resuspended in fresh lysis buffer. Half of this LD suspension is used for TAG measurement. The other half is incubated with SDS lysis buffer (10% final) at 37°C for 30 min with vortexing and then centrifuged for 10 min, after which the final LD fraction is harvested. Portions of each fraction are loaded on SDS-PAGE gels and

then immunoblotted for perilipin and HSL. This confirmed that more than 95% of perilipin is removed from the LD and present in the infranatant fraction. The infranatant fraction is immunoprecipitated using the FLAG-Tagged protein immunoprecipitation kit (Sigma) at 4°C for 24 h with rotation. Immunoprecipitates are eluted from the pelleted anti-FLAG agarose beads by boiling in SDS-PAGE sample buffer containing 2-mercaptoethanol. The reducing conditions are sufficient to break the DTSSP-induced protein crosslinks. Eluted samples are electrophoresed and immunoblotted for the LD-associated proteins, perilipin and HSL. To assess potential non-specific crosslinking of perilipin with membrane or cytosolic proteins, immunoblots are also probed with corresponding antibodies (e. g. toward clathrin, adiponectin).

EVALUATION

With respect to the phosphorylation-dependent mechanism by which perilipin promotes lipolysis, the assay monitoring the interaction of HSL and perilipin reveals that the lipolytic action(s) of LD-associated HSL require a novel event(s) mediated by PKA-dependent perilipin phosphorylation. The precise nature of this event(s) remains to be elucidated. However, it is likely to involve conformational changes in perilipin that bring LD-associated HSL into proximity with stored neutral TAG. The effected LD-associated HSL presumably includes the pool of HSL that is prepositioned at the LD in the basal state (Moore et al. 2005, Morimoto et al. 2001, Brasaemle et al. 2004). The crosslinking studies suggest that PKA-dependent phosphorylation of perilipin alters the spatial relationship between perilipin and LD-associated HSL in such a way as to facilitate close-range interaction between the two (or between HSL and a perilipin-associated moiety). This interaction may facilitate access of LD-associated HSL to stored neutral TAG, thereby initiating lipolysis. Recently, chronic PKA-dependent phosphorylation of perilipin A has been shown to induce LD remodeling (i. e. fragmentation and dispersion) independently of lipase action (Marcinkiewicz et al. 2006). This suggests that this remodeling promotes lipolysis and supports the concept that PKA-dependent phosphorylation induces dramatic conformational changes in perilipin.

Thus, perilipin seems to act as the critical component of a scaffold that stabilizes LD structure and composition for optimal TAG storage and regulated lipolysis (Moore et al. 2005, Brasaemle et al. 2004). Compounds/drug candidates interfering with lipolysis may directly modify the perilipin-HSL interaction without

affecting perilipin phosphorylation. Those compounds could be identified using the crosslinking assay.

K.6.1.13

Triacylglycerol (TAG) Lipases (HSL, ATGL) Activity

GENERAL CONSIDERATIONS

A direct effect of a compound on the activity of HSL rather than its translocation can be assayed in a cell-free system consisting of emulsified TAG rather than intact LD (see K.6.1.12.1). Lipases play a key role in human lipid metabolism as they degrade dietary nutritional as well as intracellular stored lipids and thereby initiate and regulate the release of FFA into the serum. Therefore, they represent potential targets for the development of drugs aimed at obesity, diabetes and atherosclerosis (for a review see Petry et al. 2004). Pancreatic lipase (PL), gastric lipase (GL) and HSL are responsible for the cleavage of dietary and stored lipids, respectively, to fatty acids and glycerol (and MAG) in the stomach and duodenum (GP; for a review see Lengsfeld et al. 2004), enterocytes (PL; for a review see Tiss et al. 2004) and adipocytes (HSL), which finally will appear in the blood stream directly or as lipoproteins after passage across and esterification/assembly into lipoproteins (chylomicrons) in enterocytes. In addition, lipases are responsible for the degradation of lipoproteins (TAG in chylomicrons) in the liver by hepatic lipase or of lipoproteins (VLDL, LDL from the liver, chylomicrons from enterocytes) in muscle and adipose tissue endothelium by lipoprotein lipase (LPL) and endothelial lipase (EL; for a review see Badellino et al. 2004). The activities of LPL and HSL are under tight hormonal control by a complex interplay of transcriptional and post-transcriptional mechanisms.

The rate-limiting role of HSL in the breakdown of adipose tissue TAG, postulated for decades (Fredrikson et al. 1981, Holm 2003, Kraemer and Shen 2002, Yeaman 2004, Osterlund 2001) has been challenged by the data from HSL knockout mice (Hämmerle et al. 2002, Osuga et al. 2000, Wang et al. 2001, for a review see Raben and Baldassare 2005). Catecholamine-induced lipolysis is abrogated, but residual basal lipolysis is observed in adipocytes from HSL-null mice, which surprisingly display a non-obese phenotype and the accumulation of DAG in their adipose tissues. These data suggest the existence of non-HSL lipases in adipose tissue (of rodents, at least), that preferentially hydrolyze the first ester bond of the TAG molecules. To search for such TAG lipases, gene and protein databases for murine and human pro-

teins with structural homologies to known lipases, i. e. the GX SXG motif for serine esterases and the α/β -hydrolase folds, have been screened. Candidates have been analyzed for TAG-hydrolase activity and expression in mouse adipose tissues and during differentiation of 3T3-L1 adipocytes. Only one previously undescribed enzyme fulfilled these requirements and has been named adipocyte TAG lipase (ATGL), desnutrin, or iPLA2 ζ (Zimmermann et al. 2004, Jenkins et al. 2004, Villena et al. 2004). The 260 amino-terminal amino acids of this 54-kD protein contains a predicted esterase domain in the α/β -hydrolase fold as well as a GX SXG site with a putative active serine (amino acid 47). Moreover, a “patatin” domain is present in the same domain. Patatin domain-containing proteins have been shown to exert acyl-hydrolase activity on phospholipid, MAG and DAG substrates and is thought to be involved in the ATP and acyl-CoA-independent transacylation reaction. Consistent with this, ATGL catalyze the CoA-independent transfer of oleate from a mono-olein donor to a mono- or diolein acceptor, which generates DAG and TAG, respectively. Using antibodies directed against ATGL, it has been suggested that ATGL is responsible for 75% of the cytosolic acylhydrolase activity in white adipose tissue of HSL-deficient mice. ATGL could therefore participate together with HSL in adipose tissue lipolysis. Although HSL catalyzes the rate-limiting step in TAG hydrolysis, the major physiological substrate for this enzyme is DAG, not TAG. Hämmerle and coworkers generated ATGL-deficient mice (Haemmerle et al. 2006) and thereby confirmed that ATGL is the rate-limiting lipase for the initiation of TAG catabolism in adipose and many non-adipose tissues. The association of ATGL deficiency with increased glucose tolerance, increased insulin sensitivity, and increased respiratory quotient during fasting provides compelling evidence that the decreased availability of FFAs promotes the use of glucose as metabolic fuel despite the presence of massive amounts of fat in adipose tissue and muscle. The inability to mobilize these fat stores leads to energy starvation, resulting in reduced energy expenditure, a decline in body temperature and premature death when ATGL knockout mice are stressed by cold temperature or food deprivation. Thus, ATGL plays a crucial role in energy homeostasis in mice. The observations that ATGL contributes to adipocyte lipolysis in human adipose tissue (Kershaw et al. 2006) and that genetic variation in the human ATGL gene is associated with plasma FFA, TAG and type II diabetes suggest that ATGL may be of similar relevance in humans. However, by comparing human adipose

tissue gene expression of HSL and ATGL as well as total triolein lipase activity and lipolytic capacity in adipocytes from lean vs. obese human subjects and in addition the tri- and diglyceride hydrolase activities of Cos-7 cell transfected with HSL and ATGL cDNAs, Mairal and coworkers (2006) found evidence for a down-regulation by body fat and for association with *in vitro* hydrolysis of triolein by HSL rather than ATGL. Moreover, their data argue for a predominant role of HSL as both tri- and diglyceride-hydrolysing lipase in human adipocytes. The findings reinforce the necessity for the use of appropriate human cellular assay systems for the identification and characterization of compounds/drug candidates interfering with lipid metabolism, in general, and lipolysis, in particular.

PURPOSE AND RATIONALE

Since neutral lipases are water-soluble enzymes hydrolysing insoluble long-chain TAG substrates and sometimes, in addition, phospholipids to a varying degree (LPL, EL), the cleavage reaction has to occur at the lipid-water interface (Petry et al. 2005, Hide et al. 1992, Verger 1997, Schmid and Verger 1998). This distinguishes lipases from other hydrolytic enzymes and makes them unique target proteins with regard to selectivity and mode of inhibition. The insolubility of neutral TAG has several striking consequences. Both in the serum compartment and in the cytoplasm of cells neutral lipids are never in direct contact with the aqueous milieu. They are organized as high-molecular-mass aggregates in emulsified droplets consisting of an interior TAG core and surrounded by a monolayer shell of phospholipids and embedded proteins (cytoplasmic LD and serum lipoproteins). Alternatively, lipids are organized in smaller complexes bound to serum or intracellular fatty acid/lipid-binding proteins, such as albumin and caveolin. Lipases interact with these special substrate complexes or “super-substrates” using hydrophobic domains which are exposed/unmasked upon contact as a consequence of substrate-induced conformational change, which sometimes has been called “interfacial activation” (Petry et al. 2005, Hide et al. 1992, Verger 1997, Schmid and Verger 1998). The apparently “two-dimensional” reaction of lipases does not simply follow Michaelis-Menten kinetics of enzyme reactions in aqueous milieu and critically depends on the quality of the interface. In PL the free access of lipidic substrates to the catalytic site is hampered by a so-called lid domain. This blockade is released upon interaction of lipase with the water-lipid interface inducing a conformational change. However, there is considerable evidence that HSL lacks a sim-

ilar lid domain (Ben Ali et al. 2004). The authentic, i. e. substrate-specific, measurement of lipase activity as well as the development of reliable lipase assay systems has to take these unique features into account.

Beisson and co-workers (2000) critically reviewed the lipase assay systems used most widely so far. The most sensitive and reliable assays are discontinuous “time-stop” assays using radiolabeled emulsified TAG or phosphoglycerides as substrates. The substrate has to be separated from the product by chromatographic methods or partitioning. Enzymatic fluorescence- or bioluminescence-based or chromatographic methods for determination of the unlabeled products of lipolysis are highly sensitive but are discontinuous, which is also true for even more sensitive radioactive methods. Titrimetric and potentiometric assays are continuous, but are relative insensitive and may interact with acidic or basic assay constituents, such as test compounds. To overcome these handicaps, Beisson and coworkers (1999) have developed an elegant continuous lipase assay using naturally occurring fluorescent TAG isolated from *parinari glaberrimum*. Synthetic octadeca-9,11,13,15-tetronic-3hydroxy-octadecyloxypropylester, a 1 acyl-2 alky glyceride from *parinaric acid*, represents as DAG analog an efficient substrate for HSL, but it is too sensitive toward oxidation to be used under routine conditions. An alternative ultraviolet spectrophotometric assay has been introduced by the same group which is less sensitive to oxidation using TAG from *aleutris fordii* seeds (Pencreac’h et al. 2002).

Whereas continuous assays based on soluble fluorescent substrates are frequently and successfully being used for esterases, they have rarely been described for lipases. Some of these soluble substrates have been proposed for the measurement of lipolytic activity (Hendrickson 1994, Meshulam et al. 1992). These assay systems turned out to be simple, easy-to-handle and of sufficient sensitivity. But one has to keep in mind, that they do not account for the special features of the lipase-substrate interaction and therefore do not discriminate lipases from esterases. In consequence, lipase activity may be underestimated or even remain undetected, in particular in case of assays being performed with crude enzyme sources, such as body fluids or tissue samples. Moreover, the use of water-soluble substrates may lead to gross misinterpretations of the inhibitory profiles of compounds. Consequently, lipidic substrates resembling the native substrate with regard to chain length and amphiphilic nature, equipped with appropriate fluorophores, should be applied whenever feasible.

Importantly, even huge fluorophores, such as BOD-IPY (Meshulam et al. 1992), rhodamine (Agmon et al. 1993) or pyrene (Scholze et al. 1999) coupled to lipase substrates apparently do not interfere with their cleavage by lipolytic enzymes. Thus, the nature of the fluorescence label does not seem to be very critical. In general, it should be as small and of hydrophobic character as possible to guarantee optimal interaction with the lipase, should not interact with coloured compounds and should be insensitive toward oxidation. For these reasons, the *p*-nitrobenzodifurazan (NBD) moiety has often been chosen as fluorescence label. NBD is a relative small fluorophore and is well accepted by various lipid-handling enzymes/proteins without grossly influencing their recognition and catalytic mechanisms. For instance, NBD-labelling has been used previously for determination of phospholipase A₂ activity in a time-stop assay design (Dagan and Yedgar 1987, Wittenauer et al. 1984). Müller and coworkers (1997) have shown, that 12-(7-nitrobenzo[1,2,3]oxadiazol-4-ylamino)dodecanoic acid (NBD-FA) is taken up by adipocytes and incorporated into TAG in insulin-sensitive fashion. This demonstrates acceptance of NBD-modified fatty acid and lipid precursors as substrates by lipid-handling enzymes, in general.

Starting from NBD-FA a non-soluble lipase substrate, NBD-MAG, has been introduced which upon presentation in mixed phospholipid-NBD-MAG micelles represents a “super-substrate” and is well accepted by a number of neutral lipases. Incorporation of NBD-MAG into mixed micelles and subsequent release of NBD-FA from the micelles in course of lipase action can easily be followed by a change in extinction thereby enabling the design of an assay system for a variety of neutral lipases in crude tissue samples on a routine basis.

PROCEDURE

Preparation of Rat Adipocyte Extract

Adipocytes are isolated from epididymal fat pads of Wistar rats by digestion with collagenase and subsequent separation from undigested tissue using a nylon web as described (see K.5.1.2). Cells obtained from 10 rats are washed three times with 50 ml each of homogenization buffer (25 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 µg/ml each of leupeptin, antipain and pepstatin) by flotation (500 × g, 2 min, 25°C, swing-out rotor), suspended in 10 ml of homogenization buffer and then homogenized by 10 strokes at 1500 rpm in a loosely fitting Teflon-in-glass homogenizer (15°C). The homogenate is centrifuged (3,000 × g, 10 min, 4°C). The infranatant be-

low the fat layer is aspirated and recentrifuged. This procedure is repeated three times for complete removal of residual lipid left at the top after the centrifugations. The final infranatant is centrifuged ($48,000 \times g$, 45 min, 4°C). The resulting fat-free supernatant is mixed with 1 g of heparin-Sepharose (washed five times with 25 mM Tris/HCl (pH 7.4), 150 mM NaCl), incubated at 4°C for 1 h (under head-to-end rotation of the vial) and then centrifuged ($1,000 \times g$, 10 min, 4°C). The supernatant is adjusted to pH 5.2 and incubated (30 min, 4°C). The precipitates are collected by centrifugation ($25,000 \times g$, 10 min, 4°C), suspended in 2.5 ml of 20 mM Tris/HCl (pH 7.0), 1 mM EDTA, 1 mM DTT, 70 mM NaCl, 13% sucrose, $10 \mu\text{g/ml}$ each of leupeptin, antipain and pepstatin and finally dialyzed (20 h, 4°C) against $3 \times 500 \text{ ml}$ of 25 mM Tris/HCl (pH 7.4), 50% glycerol, 1 mM EDTA, 1 mM DTT and $10 \mu\text{g/ml}$ each of leupeptin, antipain and pepstatin. Adipocyte extract is frozen in liquid N_2 and stored at -70°C for up to four weeks.

Preparation of Tissue Extracts

The tissues are surgically removed and washed in PBS containing 1 mM EDTA. Homogenization is performed on ice in lysis buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, $20 \mu\text{g/ml}$ leupeptin, $2 \mu\text{g/ml}$ antipain, $1 \mu\text{g/ml}$ pepstatin) using a ultraturax. The infranatants are obtained after centrifugation ($20,000 \times g$, 90 min, 4°C). The reaction is performed in a water bath at 37°C for 60 min with 0.1 ml of substrate and 0.1 ml of infranatant.

K.6.1.13.1

Method Based on Fluorescently Labeled Monoacylglycerol (NBD-MAG)

PROCEDURE

Preparation and Immunoprecipitation of HEK293 Cell

Lysates Ectopically Expressing Lipases

Expression constructs encoding only the open reading frame of the relevant lipase with or without epitope tags are constructed by subcloning PCR amplification products into a mammalian expression with CMV promoter or into the Gateway entry vector followed by recombination into the Gateway destination vector. Each expression vector is used to transiently transfect HEK293 cells. HEK293 cells are grown in DMEM supplemented with 10% FCS at 37°C in an atmosphere of 5% CO_2 . 10-cm tissue culture dishes with 90% confluent HEK293 cells are transfected using Lipofectamine2000 (Invitrogen). 72 h after transfection, cells are washed with ice-cold TBS and harvested. Cells are

pelleted by centrifugation ($1,000 \times g$, 5 min) and stored at -80°C until lysate preparation. For this, frozen cells are suspended in 1 ml of lipase reaction buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.53% sodium taurodeoxycholate, 1.33 mM CaCl_2) containing the complete proteinase inhibitor mix. The resulting suspensions are then sonicated on ice with 4 bursts of 10 s from a probe sonicator. Homogenized lysate is centrifuged ($1,000 \times g$, 10 min) for removal of cell debris. $50\text{-}\mu\text{l}$ portions of the resulting cleared lysates are used for direct activity measurements. For immunoprecipitation, portions are supplemented with anti-V5 mouse monoclonal antibody ($7.2 \mu\text{g}$) and incubated at 4°C overnight with tumbling. Next, $20 \mu\text{l}$ of protein A-bead suspension is added and tumbled at 4°C for 2 h. Beads are then pelleted with gentle centrifugation, washed four times with $900 \mu\text{l}$ of lipase reaction buffer followed by resuspension in $100 \mu\text{l}$ of lipase reaction buffer.

Activity Measurement

Since HSL cleaves MAG in addition to DAG and TAG albeit a rather low rate, Petry and coworkers (2005) introduced the use of NBD-MAG as substrate for a convenient, non-radioactive and homogeneous activity assay for HSL. For preparation of the NBD-MAG substrate, $41.5 \mu\text{l}$ of a PC solution (6 mg/ml in chloroform), $83.5 \mu\text{l}$ of a PI solution (6 mg/ml in chloroform) and $100 \mu\text{l}$ of a NBD-MAG solution (10 mg/ml in chloroform) are added to plastic scintillation vials are dried over a stream of N_2 and stored until use at 4°C for up to three days. The dried lipids are then re-suspended in 20 ml of 25 mM Tris/HCl buffer (pH 7.4), 150 mM NaCl and then subjected to an ultrasonic treatment in a ice bath using a Branson Sonifier (type II, standard microtip; $2 \times 1 \text{ min}$ at a setting 2 followed by $2 \times 1 \text{ min}$ at a setting 4 with 1-min intervals). During the ultrasonic treatment, the color of the substrate suspension shifts from yellow (maximum absorbance at 481 nm) to pink (maximum absorbance at 550 nm). It is used after a period of 15 min (minimum) to up to 2 h (maximum). To start the assay procedure, $180 \mu\text{l}$ of NBD-MAG substrate solution are warmed up to 30°C , supplemented with either $30 \mu\text{l}$ of an adipocyte extract (appropriately diluted with, 25 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT containing 0.1% BSA) or $20 \mu\text{l}$ of recombinant human HSL in the wells of 96-well microtiter plates.

EVALUATION

The optical density (OD) at 481 nm is recorded continuously at regular time intervals (from 1 to 30

min) using a microplate scanning spectrophotometer. Buffer alone is used as a control experiment. Alternatively, the products generated in the reaction mixture are analyzed by TLC. For this purpose 200 μ l of the reaction mixture are transferred into 2-ml reaction vials and supplemented with 1.3 ml of a methanol/chloroform/heptane (10/9/7, by vol.) and then with 0.4 ml of 0.1 M HCl. After intense vortexing, phase separation is initiated by centrifugation ($800 \times g$, 20 min, 25°C), 200- μ l aliquots of the lower organic phase are removed, then dried under vacuum (SpeedVac evaporator) and finally suspended in 50 μ l of tetrahydrofuran (THF). Samples of 5- to 10- μ l are separated by TLC on silica gel Si-60 plates using diethylether/petrol ether/acetic acid (78/22/1, by vol.) as elution solvent system. The amount of NBD-FA acid released is evaluated by fluorescence imaging using a phosphorimager (Molecular Dynamics, storm 860 and ImageQuant software) with an excitation wavelength of at 460 nm and emission wavelength of at 540–550 nm.

K.6.1.13.2

Method Based on Fluorescently Labeled TAG

For substrate preparation, NBD-TAG (total mixture of NBD-MAG/DAG/TAG) are extracted with chloroform/heptane from adipocytes, which had been incubated with NBD-FA in the presence of insulin, and purified as described (Müller et al. 1997). For 4 ml of substrate emulsion, 2 mg of dried NBD-TAG is emulsified with 0.2 mg of phosphatidylcholine/phosphatidylinositol, 3/1, w/w) in chloroform. After evaporation of the solvent (SpeedVac), 2 ml of 0.1 M potassium phosphate (pH 7.0) is added and the substrate is sonicated (4×1 min, Branson Sonifier 250, setting 2–3, 22°C). Subsequently, 1.6 ml of potassium phosphate is added and the sonication is repeated on ice. After the second sonication, 0.4 ml of potassium phosphate containing 10% BSA is added. 100 μ l of substrate emulsion is mixed with 100 μ l of rat adipocyte extract as a source for crude HSL (prepared as above) in homogenization medium and incubated (1 h, 30°C). The digestion is terminated by addition of 400 μ l of chloroform/methanol (3/1; by vol.) and analyzed as described above for cell-free lipolysis (see K.6.1.11).

K.6.1.13.3

Method Based on Radiolabeled Trioleoylglycerol (TOG)

For assaying trioleoylglycerol (TOG = triolein) cleavage, the TOG droplet emulsion (final conc. 1.7 mM) is

prepared by mixing 25–50 μCi glycerol-tri[^3H]oleate (in toluene/ethanol, 1/1), 6.8 μmol TOG (in chloroform), 0.6 mg PC and 0.2 mg PI, subsequent drying over a stream of N_2 and final suspending in 2 ml of 0.1 M KP_i (pH 7.0) under ultrasonic treatment (Branson sonifier B-12, standard microtip, setting 2, 2×1 min with 1-min intervals on ice). After addition of 1 ml of 0.1 M KP_i (pH 7.0) and ultrasonic treatment (see above, 4×30 sec with 1-min intervals on ice), the TOG substrate suspension is supplemented with 1 ml of 20% BSA (in 0.1 M KP_i) and stored until use at 4°C (for up to two weeks). The reaction is started by addition of 10 μ l of rat adipocyte extract (appropriately diluted with 20 mM M KP_i , pH 7.0, 1 mM EDTA, 1 mM DTT, 0.02% BSA, 10 $\mu\text{g}/\text{ml}$ of each leupeptin, pepstatin and antipain) to 10 μ l of TOG suspension. After incubation (30 min, 37°C), the reaction is terminated by addition of 325 μ l of methanol/chloroform/heptane (10/9/7, by vol.). Phase separation is initiated by addition of 105 μ l of 0.1 M K_2CO_3 , 0.1 M boric acid (pH 10.5), intense vortexing and centrifugation ($8,000 \times g$, 15 min). 150 μ l of the upper aqueous phase is transferred into scintillation vials and supplemented with 5 ml of scintillation cocktail (Beckman Ready Safe) for measurement of radioactivity by liquid scintillation counting. This method has been used by Vertesy and coworkers (2002) for the identification of novel inhibitors of HSL with putative anti-diabetic activity from natural sources, such as funghi.

K.6.1.13.4

Method Based on Radiolabeled Tributyrin

For assaying tributyrin cleavage, 10 μ l of [^{14}C]tributyrin (20 mM, 0.1 μCi , in ethanol) are dried, then suspended in 25 μ l of 0.1 M KP_i (pH 7.25), 140 mM NaCl, 1 mM DTT, 0.2 mM EDTA and finally incubated (30 min, 37°C) with 25 μ l of rat adipocyte extract (appropriately diluted with the same buffer). The reaction is terminated by the addition of 325 μ l of methanol/chloroform/heptane (10/9/7, by vol.) and 125 μ l of 0.1 M NaOH and subsequently processed and described as above (see K.6.1.13.3).

K.6.1.13.5

Method Based on Resorufin Ester

The assay uses 1,2-dilauryl-rac-glycero-3-glutaric acid (6'-methylresorufin) ester (DGGR) as a substrate (Lehner and Verger 1997). DGGR is cleaved by lipase, resulting in an unstable dicarboxylic acid ester that is spontaneously hydrolyzed to yield glutaric acid and

methylresorufin, a bluish-purple chromophore with peak absorption at 581 nm. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Hydrolysis of DGGR (final conc. 100 μ M) is determined at 37°C in a final volume of 1 ml. For this, substrate solution is prepared by mixing 40 μ l of the ester (2 mg/ml dioxane) with 160 μ l of 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, and 0.05% TX-100. 790 μ l of 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.53% sodium taurocholate and 1.33 mM CaCl₂ is added, and the reaction is started by addition of 10 μ l of rat adipocyte extract. The release of resorufin is monitored at 572 nm. For high-throughput screening of compounds/drug candidates activating or inhibiting lipases, 10 μ l of whole-cell lysate or immunoprecipitation beads is added to the wells of a 96-well plate. After adjustment of the samples to 125 μ l in lipase reaction buffer, 125 μ l of reaction buffer containing DGGR is added (final conc. of 36 μ g/ml in a final assay volume of 250 μ l per volume). After mixing, OD 581 is monitored at 5 min-intervals for 2 h to assess lipase activity. This procedure has been used by Lake and coworkers (2005) for the analysis of the expression, regulation, and triglyceride hydrolase activity of the adiponutrin family members.

K.6.1.13.6

Method Based on *p*-Nitrophenylbutyrate

For assaying *p*-nitrophenylbutyrate (PNPB) cleavage according to the procedure described by Holm and Osterlund (1999) with modifications from Soni and coworkers (2004), 10 μ l of 2 mM PNPB (in acetonitrile) are added to 890 μ l of 0.1 M KP_i (pH 7.25), 140 mM NaCl, 1 mM DTT, 0.2 mM EDTA and then incubated (10 min, 37°C) in the presence of 100 μ l of rat adipocyte extract (appropriately diluted with the same buffer) in the absence or presence of 0.01% TX-100. The reaction is terminated by adding 3.25 ml of methanol/chloroform/heptane (10/9/7), vigorously vortexed for 10 s, and then centrifuged (800 \times g, 20 min, 21°C). The upper phase is collected and thereafter subjected to incubation at 42°C for 2 min. The absorbance of the released *p*-nitrophenol (extinction coefficient of 12,000) is measured at 405 nm.

K.6.1.13.7

Method Based on Potentiometry

For a potentiometric assay, the hydrolysis of short- and medium-chain TAG is determined at pH 8.0 and 37°C with a pH-stat (TTT 80 Radiometer) by titrating liber-

ated fatty acids with 0.1 N NaOH. The standard mixture using tributyrin contains 14.9 ml of 150 mM NaCl and 100 μ l of tributyrin (final vol. 15 ml). With TOG, the assay mix is composed of 14.5 ml of 150 mM NaCl, 2 mM sodium taurodeoxycholate, 0.1 mg/ml BSA and 0.5 ml of TOG.

K.6.1.14

Neutral Cholesterylester Hydrolase Activity

HSL possesses pronounced neutral cholesterylester hydrolase activity in addition to its neutral TAG hydrolase activity in contrast to ATGL, which exhibits the latter, exclusively (Zimmermann et al. 2004, Jenkins et al. 2004). Consequently, assaying cholesterylester hydrolase activity can be used to discriminate between HSL and ATGL activity in crude cell lysates. Cholesterylester hydrolase is measured according to the procedure described by Holm and Osterlund (1999) with modifications from Soni and coworkers (2004) and Ben Ali and coworkers (2005). 1.17 mg of cholesteryl oleate, including 2×10^7 dpm of [¹⁴C]cholesteryl oleate, plus a phospholipid mixture (71.4 μ l, including phosphatidylcholine [15 mg/ml] and phosphatidylinositol [5 mg/ml], dissolved in chloroform) are placed in a 4-ml glass vial. Solvents are evaporated under a gentle stream of N₂. 2 ml of 0.1 M potassium phosphate (pH 7.5) is added. After incubation (10 min, 37°C), the mixtures are sonicated (twice for 1 min each, separated by a 1-min interval). An additional 1 ml of 0.1 M potassium phosphate (pH 7.5) is added and incubated (10 min, 37°C). The mixtures are sonicated again (four times for 30 s each on ice, separated by 30-s intervals). After sonication, 1 ml of 20% fatty acid-free BSA prepared in 0.1 M potassium phosphate (pH 7.5) is added. For each assay, 100 μ l of substrate is mixed with 100 μ l of sample and incubated (30 min, 37°C). The reactions are terminated, extracted and counted for radioactivity as described above for the method based on radiolabeled TOG.

K.6.1.15

Lipoprotein Lipase (LPL) Activity

For assaying LPL according to Dichek and coworkers (1993) and Iverius and coworkers (1985) with modifications, substrate is prepared by evaporation of 5 mg of unlabeled TOG together with 50 μ Ci glycerol-tri[³H]oleate and 0.24 mg PC from the solvent, toluene, under a stream of N₂. The dried lipids are emulsified in 1 ml of 1 M Tris/HCl (pH 8.5), 0.1 M NaCl and 2.5% BSA in the absence or presence of 1 M

NaCl and in the presence of 8% heated rat serum under sonication (Branson Sonifier B-12, standard microtip, setting 2, 10 s on, 10 s off, for 10 cycles on ice). Affinity-purified LPL from bovine milk in 15 μ l of 10 mM Tris/HCl (pH 7.4), 0.5 mM DTT are incubated (60 min, 37°C) with 5 μ l of substrate emulsion. Reactions are terminated by addition of 325 μ l of methanol/chloroform/heptane (10/9/7, by vol.) and subsequently of 105 μ l of 0.1 M K₂CO₃, 0.1 M boric acid (pH 10.5). After vortexing, the phases are separated and 100- μ l portions of the upper aqueous phase are mixed with 5 ml of scintillation cocktail (Beckman, Ready Safe) and counted for radioactivity. LPL activity is determined by subtracting the non-LPL-dependent lipolytic activity (presence of 1 M NaCl) from the total activity (absence of 1 M NaCl).

K.6.1.16

Analysis of Lipolysis Products

TAG Hydrolysis Rate

For the quantitation of DAG and MAG formed by the TAG lipase actions, the reaction is terminated by adding 1 ml of chloroform/methanol (2/1, by vol.) containing 1% acetic acid, oleic acid (10 μ g/ml) and standards for monooleoylglycerol (MOG) and dioleoylglycerol DOG (10 μ g/ml, sn-1,2 and sn-1,3). The mixture is vortexed vigorously three times over 15 min. After centrifugation (4,000 \times g, 10 min), 0.5 ml of the lower phase is collected and evaporated under nitrogen. The lipid pellet is dissolved in chloroform and applied onto TLC (Merck, silica gel 60) with chloroform/acetone/acetic acid (96/4/1, by vol.) as solvent. Lipids are visualized with iodine vapor and the bands corresponding to MOG, DOG and TOG and oleic acid are cut out. The comigrating radioactivity is determined by liquid scintillation counting.

Alternatively, for determination of the rate of DAG formation, the lipolysis reaction is terminated by adding 25 μ l of 1 M HCl and 1 ml of hexane/isopropyl alcohol (3/2, by vol.) containing oleic acid (10 μ g/ml) and standards for MOG, DOG (sn-1,2 and sn-1,3, Sigma) and TOG (10 μ g/ml each). The mixture is vortexed vigorously three times over a period of 15 min. After centrifugation (4,000 \times g, 10 min), 0.4 ml of the upper phase is collected and evaporated under nitrogen. The lipid pellet is dissolved in chloroform and loaded onto a TLC plate (Merck, silica gel 60). The TLC is developed with chloroform/acetone/acetic acid (96/4/1) as solvent. The lipids and FFA are visualized with iodine vapor, and the bands corresponding to MOG, DOG and TOG and oleic acid are cut out.

The comigrating radioactivity is determined by liquid scintillation counting, and the molar concentrations of the products are calculated.

EVALUATION

The specific TAG hydrolase activity (TAG to DAG conversion) is calculated from the total AG lipolytic activity and the DAG formed during the reaction (considering that MAG does not accumulate) by the equation given by Hämmerle and coworkers (2002).

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K.6.1.17 Affinity Labeling of TAG Lipases

PURPOSE AND RATIONALE

Compounds which interfere with lipolysis by direct blockade of TAG cleavage can be directed to various lipases in differential fashion. So far, in addition to HSL, ATGL, GS-2 and adiponutrin have been identified as putative TAG degrading lipases in rodent adipocytes. Their differential targeting by compounds/drug candidates can be analyzed by activity tagging (for a review see Petry et al. 2004). A fluorescence technology for selectively screening for lipolytic enzymes developed by Schmidinger and coworkers (2005) is based on the so-called click chemistry utilizing the copper(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction (Speers and Cravatt 2004a and b, Wang et al. 2003). Lipase- and esterase-mediated hydrolysis of acyl esters is based on a mechanism involving a nucleophilic serine, which in most cases is part of a catalytic triad with histidine and aspartate. In the first reaction step, the active serine attacks the carbonyl group of the scissile fatty acid to give a tetrahedral transition state. This intermediate state is cleaved to yield the free fatty acid, the alcohol component and the nucleophilic serine. Although many lipases show strong structural and sequential similarities (Pleiss et al. 1998, Arpigny and Jaeger 1999), their substrate and stereospecificities can

vary significantly (Martinelle et al. 1995). It is known that *p*-nitrophenylesters of alkylphosphonic acids irreversibly and stoichiometrically react with the nucleophilic serine of lipases and esterases, freezing the reaction at the point of the tetrahedral transition state. In the past, they have been applied to the determination of serine hydrolase activity by using different approaches (Oskolkova et al. 2003, Rotticci et al. 2000). If fluorescent inhibitors are used, the tagged enzyme becomes visible (Mannesse et al. 1995). Thus, it can be detected and quantified on the basis of its fluorescent signal (Scholze et al. 1999). Fluorescently labeled alkyl phosphonates detect active enzymes in electrophoretically pure proteins and in complex proteosome samples (Speers and Cravatt 2004a, Adam et al. 2002, Greenbaum et al. 2002).

PROCEDURE

Activity tagging of lipases contained in adipocyte tissue can be performed according to the protocol of Birner-Grünberger and coworkers (2005). For this, gonadal fat pads (white adipose tissue) of fed and fasted mice are surgically removed and washed in PBS. Homogenization is performed on ice in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin) using a motor-driven Teflon-in-glass homogenizer (8 strokes, 1,500 rpm). Cell debris is removed by centrifugation (1,000 × *g*, 15 min) to obtain cytoplasmic extracts. Protein concentrations is determined using the BIORAD protein assay based on the method of Bradford. 50 µg of protein sample dissolved in lysis buffer, 10 µl of 10 mM Triton X-100 in methanol (final conc. 1 mM) and 20 µl of activity tag (NBD-labeled single-chain phosphonic acid ester, TAG phosphonates and cholesteryl phosphonates which react with the nucleophilic serine in the active site of lipolytic enzymes due to the active phosphonate moiety with the good leaving PNPB group and then remain covalently attached leading to irreversible inactivation of the lipases) dissolved in methanol (1 nmol/10 µl, final conc. 20 µM) are mixed and the organic solvent is evaporated under a stream of argon. 100 µl of protein sample (0.5 mg protein/ml) is added and the resultant mixture is incubated (2 h, 37°C) under light protection. Proteins are precipitated in 10% ice-cold TCA (1 h, on ice) and collected by centrifugation (10,000 × *g*, 15 min, 4°C). The pellet is washed once with ice-cold acetone and resuspended in 1D sample buffer (20 mM KH₂PO₄, 6 mM EDTA, 60 mg/ml SDS, 100 mg/ml glycerol, 0.5 mg/ml bromophenol blue, 20 µl/ml mercaptoethanol, pH 6.8) or 2D sample buffer (7 M urea,

2 M thiourea, 4% CHAPS, 60 mM DTT, 2% Pharmalyte pH 3–10, 0.002% bromophenol blue). SDS-PAGE is performed in a Tris/glycine buffer system by aligning proteins (50 or 100 µg protein/lane) in a 5% stacking gel and separating them in a 10% resolving gel at 20–50 mA constant current. 2D-gel electrophoresis is performed in the first dimension by isoelectric focusing of 50–500 µg protein in 7 or 18 cm immobilized non-linear pH 3–10 gradients at 6.5/12 kVh and in the second dimension by SDS-PAGE on 7 or 20 cm gels as described by Gorg and coworkers (1985, 1988). Gels are fixed in 7.5% acetic acid and 10% ethanol and scanned at a resolution of 100 µm. NBD fluorescence is detected at 530 nm and an excitation wavelength of 488 nm. For visualization of the whole protein pattern, gels are stained with SYPRO Ruby following the manufacturer's instructions (Molecular Probes) and scanned at 605 nm and an excitation wavelength of 488 nm. For LC-MS/MS-analysis, the fluorescent protein spots are excised and tryptically digested according to the method by Shevchenko and coworkers (1996). Peptide extracts are dissolved in 0.1% formic acid and separated on a nano-HPLC-system. 20-µl samples are injected and concentrated on the loading column (LC packings PepMapTM C-18, 5 µm, 100 Å, 300 µm ID × 1 mm) for 5 min using 0.1% formic acid as isocratic solvent at a flow rate of 20 µl/min. The column is then switched into the nano-flow-circuit and the sample is loaded on the nano-column at a flow rate of 300 nl/min and separated using a gradient from 0.3% formic acid and 5% acetonitrile to 0.3% formic acid and 50% acetonitrile over 60 min. The sample is ionized in a Finnigan nano-ESI source equipped with NanoSpray tips and analyzed in a Thermo-Finnigan LCQ Deca XPplus iontrap mass-spectrometer. Acceptance parameters are two or more identified distinct peptides (Carr et al. 2004). Identified protein sequences are subjected to BLAST and motif search for identification of potential serine hydrolases.

EVALUATION

In pilot experiments, 50 µg of cleared cell lysate (see above) is incubated with 1 nmol of fluorescently labeled lipase inhibitor O-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)-aminoethyl-O-(*n*-hexyl)phosphonic acid *p*-nitrophenyl ester () and 1 mM TX-100 at 37°C for 2 h under shaking. Total protein is then precipitated with 10% TCA for 1 h on ice, washed once with acetone and subjected to 10% SDS-PAGE. After treatment of the gels with 10% ethanol and 7% acetic acid, the fluorescent signals are detected with laser scanner (excitation 488 nm, emission 530 nm).

K.6.1.18**Interaction of ATGL and CGI-58****PURPOSE AND RATIONALE**

In contrast to HSL, which undergoes PKA-dependent phosphorylation and translocation from the cytoplasm to LD upon β -adrenergic stimulation of adipocytes, the regulation of ATGL may rely on protein-protein interaction. It has been demonstrated by Lass and coworkers (2006) that efficient enzymic activity of ATGL depends on interaction with and activation by CGI-58. Mutations in the human *CGI-58* gene are associated with Chanarin-Dorfman Syndrome (CDS), a rare genetic disease where TG accumulates excessively in multiple tissues (Chanarin et al. 1975, Dorfman et al. 1974, Lefevre et al. 2001). CGI-58 interacts with ATGL, stimulating its TAG hydrolase activity up to 20-fold. Alleles of *CGI-58* carrying point mutations associated with CDS fail to activate ATGL. Moreover, CGI-58/ATGL coexpression attenuates lipid accumulation in COS-7 cells. Antisense RNA-mediated reduction of CGI-58 expression in 3T3-L1 adipocytes inhibits TAG mobilization. These data establish an important biochemical function for CGI-58 in the lipolytic degradation of TAG and raise the question whether defects in CGI-58 may contribute to the pathogenesis of type II diabetes and obesity. The following assays allow the analysis of the functional interaction of ATGL with CGI-58 and its modulation by compounds/drug candidates.

PROCEDURE**Preparation of ATGL**

Monkey embryonic kidney cells (COS-7, ATCC CRL-1651) are transfected using Metafectene (Biontex, Germany) as described (Zimmermann et al. 2004). For the preparation of cell extracts, cells are collected by trypsin treatment, washed three times with PBS and disrupted in buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μ g/ml leupeptine, 2 μ g/ml antipain, 1 μ g/ml pepstatin, 20 mM Tris/HCl, pH 7.0) by sonication. Nuclei and unbroken cells are removed by centrifugation (1,000 \times g, 4°C, 5 min). The expression of the His-tagged proteins is detected using immunoblotting as described (Zimmermann et al. 2004). COS-7 cells expressing His-tagged mouse ATGL are lysed in buffer C (25 mM NaPO₄, pH 7.0, 20% glycerol, 2 mM β -mercaptoethanol, 0.01% NP-40) and incubated with TALON Co²⁺ resin (BD Biosciences). The resin-cell extract suspension is then poured into a micro bio-spin column (Bio-Rad) and washed with 10 column volumes of buffer C containing 500 mM

NaCl. Recombinant His-tagged ATGL is eluted by a step-gradient of imidazole (200 mM final concentration) in buffer C. For determination of the purity of the protein, fractions are subjected to SDS-PAGE gel electrophoresis and immunoblotting.

Preparation of GST-Tagged CGI-58

The coding sequence of mouse CGI-58 is cloned into pYex4T-1 vector and transformed into the *S. cerevisiae* BY4742 (Mat α ; his3; leu2; lys2; ura3) strain. Large-scale overexpression of GST-CGI is achieved by maintaining transformed *S. cerevisiae* carrying in YNB-urea containing 0.5 mM CuSO₄ to induce copper promoter-driven expression of the fusion cassette. After induction, cells are harvested and protoplasts are generated with zymolyase and disrupted by sonication in the presence of 0.2% NP-40. The supernatant containing the GST-fusion protein is purified using glutathione-Sepharose beads. Purified GST-CGI is dialyzed overnight with 150 mM KCl, 10 mM potassium phosphate buffer (pH 7.4), and 0.01% NP-40.

Interaction Assay

For the detection of interacting proteins, ELISA plates are coated with 3 μ g GST-CGI or GST in buffer D (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM CaCl₂). The wells are blocked with 5% BSA in buffer D and incubated with 50 μ g protein/well of COS-7 cell extracts containing His-tagged ATGL in 50 mM potassium phosphate buffer (pH 7.0) in the absence or presence of compounds/drug candidates. After washing with buffer D containing 0.05% Tween 20, the mouse anti-His antibody is added in the same buffer containing 0.5% BSA. Subsequent to three further washes, horseradish peroxidase-conjugated anti-mouse antibody is added. After washing three times with buffer D containing 0.05% Tween 20, the absorbance of tetramethyl-benzidine is determined at 450 nm using 620 nm as reference wavelength. The absorbance of GST coated wells is subtracted from that coated with GST-CGI.

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K.6.1.19

Measurement of cAMP Levels

PURPOSE AND RATIONALE

Activation of lipolysis in adipocytes by β -adrenergic stimuli and its inhibition by insulin critically depends on the generation by adenylate cyclase and degradation by phosphodiesterase 3B (PDE3B), respectively (for a review see Müller and Petry 2004). The intracellular cAMP levels can be determined in adipocytes which have been exposed to isoproterenol prior to incubation with insulin/compounds/drug candidates.

PROCEDURE

400 μ l of adipocyte suspension (lipocrit 10%) are suspended in 600 μ l KRH containing 0.75% BSA, 1.5 mM glucose, and incubated in the absence or presence of lipolytic stimuli (isoproterenol, adenosine deaminase) in combination with insulin or compounds/drug candidates at 37°C in a mildly shaking water bath. 200- μ l aliquots are transferred into Eppendorf cups containing 20 μ l HClO₄ (70%), vortexed vigorously and centrifuged (12,000 \times g, 15 min, room temperature). 110 μ l of the infranatant is removed, taking care not to aspirate any of the precipitate, supplemented with 60 μ l of 1 M Tris/HCl (pH 7.5), incubated for 15 min on ice and then centrifuged (13,000 \times g, 15 min, 4°C). 150 μ l of the infranatant is removed for glycerol determination, frozen in liquid N₂ and stored at -20°C prior to determination of glycerol and cAMP levels. cAMP is determined by a modification of the protein kinase binding procedure. Samples and standards are incubated with 8 μ g of R-subunit of PKA and 175 nCi [8-³H]cAMP in a buffer composed of 50 mM Tris/HCl (pH 7.4), 4 mM EDTA in a final volume of 200 μ l for 4 h at 4°C to reach equilibrium. Charcoal suspension (30 g/l) is added and after vortexing, the mixture is centrifuged at 16,000 \times g, 2 min, 4°C). The supernatant is transferred into scintillation vials, supplemented with 5 ml water-compatible scintillation fluid

and counted for radioactivity using a liquid scintillation counter.

EVALUATION

The concentration of endogenous unlabeled cAMP in the samples is determined from a linear standard curve.

K.6.1.20

cAMP-Specific Phosphodiesterase (PDE) Activity

PURPOSE AND RATIONALE

Membrane-bound phosphodiesterase of rat epididymal adipose cells is stimulated when intact cells are exposed to insulin. The localization at the endoplasmic reticulum (corresponding to the low density microsomal fraction) and properties of the insulin-sensitive particulate cGMP-inhibitable cAMP phosphodiesterase have been described by Kono and coworkers (1975), Osegawa and coworkers (1982), Saltiel and Steigerwalt (1985), Solomon and coworkers (1986). Recently, the phosphodiesterase isoform, PDE3B, has been identified as target for phosphorylation and activation by protein kinase PKB/Akt, which is a key element of the downstream insulin signaling cascade.

PROCEDURE

The low density microsomal fraction (LDM) of primary or cultured adipocytes (pellet of a $100,000 \times g/60$ min-centrifugation of the post-mitochondrial supernatant prepared from the homogenate of adipocytes exposed to lipolytic stimuli in the absence or presence of compounds; see above) is incubated in the absence or presence of compounds at various concentrations for 20 min. For the assay of cAMP-specific PDE up to 50 μ g of LDM protein are incubated (5 min, 30°C) with 500 nM [2,8- 3 H]cAMP (100 nCi) in 50 mM Tris/HCl (pH 7.4), 0.5 mM DTT, 5 mM MgCl₂, 50 μ M PMSF in a total volume of 0.25 ml. The incubation is terminated by the sequential addition of 30 μ l of 10 mM IBMX and 120 μ l of 0.1 N HCl and heating (5 min, 95°C). After neutralization (120 μ l of 0.1 N KOH, 80 ml of 250 mM Tris/HCl (pH 7.4), 10 μ l of crude 5'-nucleotidase (5 mg/ml) (*Crotalus atrox*) is added to the mixture. The reaction (30 min, 37°C) is terminated by the addition of 60 μ l of 200 mM EDTA, 5 mM adenosine. Unreacted cAMP is removed by the addition of 1 ml of a 1:3 slurry of Dowex AG-1X8 (Bio-rad). Solutions are shaken (5 min, 4°C) and centrifuged (1,000 \times g, 5 min).

EVALUATION

Radiolabeled adenosine left in the supernatant after enzymic degradation of 5'-AMP and chromatographic removal of cAMP is determined by liquid scintillation counting. The assay is proportional to up to 100 μ g of protein and linear throughout 20 min. It monitors the putative insulin-like effects of compounds on the regulation of cAMP degradation *via* the insulin signaling cascade (or alternative pathways) as well as directly on the activity state of PDE3B depending on whether or not the compound is present during the incubation of the adipocytes or LDM.

K.6.1.21

Activity State of Protein Kinase A (PKA)

PURPOSE AND RATIONALE

The cAMP levels generated in response to β -adrenergic stimulation in the absence or presence of insulin or compounds in adipocytes determine the activity of PKA phosphorylating both HSL and perilipin which play key roles in the regulation of lipolysis in adipocytes (see K.6.1.10 and K.6.1.12.2). Thus, the PKA activity ultimately determines the lipolytic state of the adipocytes in response to insulin/compounds/drug candidates. The assay monitors the activity state of PKA in adipocytes in response to β -adrenergic stimuli in the absence or presence of insulin/compounds/drug candidates at the time point of homogenization. This activity ratio is a parameter for the portion of PKA active *in vivo* toward total cellular PKA at the time point of homogenization.

K.6.1.21.1

Radioactive Method

For determination of the activity state of PKA, 500- μ l adipocyte suspension (5×10^6 cells/ml) are transferred to 2-ml microfuge tubes, pre-cooled to 4°C, containing at final concentrations: 20 mM Tris/HCl (pH 7.4), 10 mM EDTA and the cAMP PDE inhibitor, Ro 20-1724 (100 μ M). The mixture is vortexed briefly, decanted into a 5-ml Teflon-in-glass homogenizer and homogenized with 10 strokes of the rotating (1,500 rpm) Teflon pestle. The homogenate is transferred to pre-cooled microfuge tubes and centrifuged (16,000 \times g, 10 min, 4°C). The infranatant is removed and immediately assayed for PKA activity. 25 μ l of assay medium (20 mM MOPS/KOH, pH 7.0, 10 mM MgCl₂, 0.1 mM ATP, 0.5 mg/ml histone H1, 5 mM DTT, 50 μ Ci/ml [γ - 32 P]ATP) is added to 25 μ l infranatant. After vortexing and incubation for 20 min

at 30°C, the reaction is terminated by addition of 1 ml of ice-cold 20% TCA containing 5 mM sodium pyrophosphate and incubation (1 h, 4°C). The precipitates are sedimented by centrifugation (16,000 × g, 5 min, 4°C) and, after removal of the supernatant, dissolved in 100 µl of cold 1 N NaOH and re-precipitated with 20% TCA and 5 mM sodium pyrophosphate. The precipitate is then filtered under vacuum over GF/C-glass microfiber filter that had been pre-wetted with 5% TCA, 5 mM sodium pyrophosphate. The filters are washed with 4 ml of the same medium, dried and counted for radioactivity.

EVALUATION

Each adipocyte infranatant is assayed in quadruplicate under four separate conditions: (I) with no further additions; (II) in the presence of 15 µM cAMP; (III) in the presence of 2 µM PKA inhibitor synthetic peptide and (IV) in the presence of 15 µM cAMP and 2 µM PKA inhibitor synthetic peptide. The results are expressed as corrected PKA activity ratios (I)–(III)/(II)–(IV) according to Honnor and coworkers (1985a and b) and Londos and coworkers (1985).

K.6.1.21.2

Fluorescent Method

PROCEDURE

The method based on fluorescently labeled kemptide has been introduced by Müller and coworkers (2000) and Schölch and coworkers (2004) and can be used for determination of the activity ratio in cells or tissues from rats treated with compounds/drug candidates. For the *ex vivo* experimental design, frozen tissue samples are minced under liquid N₂ in 2 ml of 25 mM MES (pH 6.0), 140 mM NaCl, 2 mmol/l EDTA, 0.5 mM EGTA, 0.25 M sucrose, 50 mM NaF, 10 mM sodium pyrophosphate, 20 mM glycerol-3-phosphate, 1 mM sodium orthovanadate, 2 µM microcystin, 1 µM okadaic acid, and protease inhibitors per gram of wet weight and then homogenized by sequential use of an Ultraturrax homogenizer (30 s, maximum speed) and a Teflon-in-glass homogenizer (tightly fitting, five strokes, 3,000 rpm, on ice). After centrifugation (500 × g, 5 min, 4°C), the infranatant below the fat layer is carefully removed, adjusted to 0.5% Triton X-100, and after incubation (30 min, 4°C) and homogenization (Teflon-in-glass, 10 strokes, 500 rpm, 4°C) centrifuged (10,000 × g, 15 min, 4°C). The supernatant is re-centrifuged (100,000 × g, 1 h, 4°C). Twenty-five microliters of the supernatant (cytosol) are incubated (30 min, 30°C) with 12.5 µl of assay buffer (200 mM

Tris/HCl, pH 7.5, 40 mM MgCl₂, 4 mM ATP, 400 µM rhodamine-labeled kemptide [PepTag A1 peptide obtained from Serva/Promega Heidelberg, Germany]) in the absence or presence of 10 µM mono-butyryl-cAMP and 50 µM heat-stable PKA inhibitor peptide (Sigma, Germany) in a total volume of 50 µl. The reaction is terminated by placing the tube into a boiling water bath for 2 min, and the mixture is stored at –20°C for further analysis. Portions of this mixture (10 µl) supplemented with 1 µl of 80% glycerol are separated by agarose gel electrophoresis (0.8% in 50 mM Tris/HCl, pH 8.0, 15 min at 100 V). Phosphorylated kemptide (net charge –1) migrating toward the anode and separated from the nonphosphorylated species (net charge +1) moving toward the cathode is visualized under UV light. For quantitative evaluation, the gel material that contained the phosphorylated band is excised (125 µl), heated (95°C), and supplemented with 75 µl of gel solubilization solution and 50 µl of glacial acetic acid, and finally its absorbance is determined at 570 nm using a 96-well plate reader (normalized for liquefied agarose containing no kemptide).

EVALUATION

The PKA activity ratio is calculated as the ratio between the absorbance in the absence and presence of cAMP corrected in each case for unspecific phosphorylation in the presence of the PKA inhibitor (which typically is < 15% of total) and reflects the portion of PKA activity (i. e., cAMP concentration) present at the time point of removal of the tissue or cell samples. Control experiments demonstrated that the activity ratio is independent of dilution of the tissue or cell homogenates (and protein concentration) from 2 g wet wt/ml to 0.03 g/ml (limit of detection) corresponding to 20–0.02 mg protein/ml and of incubation period (up to 2 h), thereby excluding formation of inactive PKA dephosphorylated holoenzyme during homogenization and subsequent assay.

K.6.1.22

PKA Catalytic Activity

PURPOSE AND RATIONALE

In addition to the regulation of the PKA activity by modulation of the cytosolic cAMP levels, insulin and insulin-like compounds/drug candidates may affect PKA by modulating its regulation *via* signaling mechanisms as provoked by insulin or by its direct allosteric activation or blockade by small molecule inhibitors.

PROCEDURE

PKA activity is assayed according to Roskoski and coworkers (1983) and Honnor and coworkers (1985a). The reaction mixture contains 25 μ l of rat adipocyte cytosol, 0.4 μ M kemptide (consensus substrate peptide), 40 mM Tris/HCl (pH 7.2), 2 mM DTT, 12.5 mM MgCl₂, 0.1 mM PMSF, 1 mM IBMX, 100 μ M [γ -³²P]ATP (1 μ Ci) with or without 1 μ M cAMP in a total volume of 100 μ l. After incubation (10 min, 30°C), the reaction mixture is chilled on ice, then supplemented with 3 ml of 75 mM phosphoric acid, 100 mM NaF, 10 mM ATP and immediately spotted on phosphocellulose filters (Whatman P18). After extensive washing with 75 mM phosphoric acid, the filters are dried and counted for radioactivity.

MODIFICATIONS OF THE METHOD

The inhibition of isoproterenol-stimulated PKA by sulfonyleureas in rat adipocytes was tested by Müller and coworkers (1994). The direct effect of tolbutamide and glyburide on the activity of PKA in rat liver cytosol was investigated by Okuno and coworkers (1988).

K.6.1.23**Protein Phosphatase (PP) Activity****PURPOSE AND RATIONALE**

Insulin inhibits lipolysis by decreasing the phosphorylation and thus the activation state of HSL in adipose tissue *via* both the inhibition of PKA and the stimulation of protein phosphatase 2A (PP2A) *via* ill-defined mechanisms. They may involve the phosphoinositol-3'-kinase (PI3K) signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway or soluble mediators of the phosphoinositolglycan (PIG) class (Vila et al. 1990; see K.6.3.6.4).

PROCEDURE

For measurement of PP2A activity, the adipocytes (3×10^7 cells) are washed once with 25 ml of ice-cold 100 mM Tris/HCl (pH 8.5), 10 mM EDTA, 25 mM DTT and immediately resuspended in 0.5 ml of 100 mM Tris/HCl (pH 7.0), 2 mM EDTA, 10 mM DTT, 0.5 mM benzamidine, 0.2 mM PMSF, 2 μ g/ml leupeptin, 5 μ g/ml pepstatin. After addition of the same volume of ice-cold glass beads, cell extracts are prepared by vigorous vortexing (5 times 5 s each with cooling intervals on ice) and centrifuged (13,000 \times g, 5 min, 4°C). The supernatant (S13) is diluted with 3 volumes of the same buffer containing 1% BSA. Protein phosphatase activity is assayed as the ability to dephosphorylate ³²P-labeled myelin basic protein (MBP). 10 μ l of S13 is added to an assay mixture (total volume 50 μ l) containing 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, and 0.2 nmol ³²P-labeled myelin basic protein (900 dpm/pmol) for 20 min at 30°C in the absence or presence of 2 nM okadaic acid. The reaction is terminated by the addition of 50 μ l of ice-cold 10% TCA. After incubation for 15 min on ice and centrifugation (12,000 \times g, 5 min, 4°C), the supernatant is neutralized with NaOH and measured for radioactivity by liquid scintillation counting (10 ml Aquasol).

tein (MBP). 10 μ l of S13 is added to an assay mixture (total volume 50 μ l) containing 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, and 0.2 nmol ³²P-labeled myelin basic protein (900 dpm/pmol) for 20 min at 30°C in the absence or presence of 2 nM okadaic acid. The reaction is terminated by the addition of 50 μ l of ice-cold 10% TCA. After incubation for 15 min on ice and centrifugation (12,000 \times g, 5 min, 4°C), the supernatant is neutralized with NaOH and measured for radioactivity by liquid scintillation counting (10 ml Aquasol).

EVALUATION

PP2A activity is determined as the difference between ³²P-radiolabeled MBP measured in the presence of okadaic acid (10 nM) corresponding to the PP2A independent phosphatase portion and radiolabeled MBP determined after uninhibited incubation corresponding to the total phosphatase activity. Under these conditions, release of ³²P_i is linear for up to 10 min. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μ mol of ³²P_i-radiolabel from labeled myelin basic protein in one min under conditions of the standard assay (Müller et al. 2000).

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K.6.2**Assays for Insulin and Insulin-Like Metabolic Activity Based on Hepatocytes, Myocytes and Diaphragms****GENERAL CONSIDERATIONS**

Despite the eminent importance of studies with primary and cultured adipocytes or adipose tissues on basis of their physiological role in the regulation of lipid and carbohydrate metabolism in humans in combination with the relative low expenditure in preparing adipocytes of high quality and number, compounds and drug candidates for future anti-diabetic and anti-obesity therapy have to be analyzed for their effects in primary and cultured hepatocytes and myocytes or liver and muscle tissues, too. In principle, the majority of the assays described above for adipocytes can be adapted for the use with hepatocytes and myocytes. However, the following selection takes into account the relative contribution of each process monitored to its role in the whole-body regulation of intermediary metabolism in the normal and disease state. Moreover, technical aspects, such as requirement for a special equipment and applicability in throughput screening assays for drug discovery were additional criteria.

K.6.2.1**Glucose Oxidation****PROCEDURE*****Cultured Myocytes and Hepatocytes***

For the measurement of glucose oxidation on basis of $^{14}\text{CO}_2$ release, varying amounts of ^{14}C glucose (0.1 mCi/ml) are used. Alternatively, glucose oxidation

is determined as the formation of ^3H water from ^3H glucose. After culture in DMEM medium in 24-well culture dishes, cells are incubated at 37°C for 2 h in $10\ \mu\text{l}$ of KRB solution containing ^3H glucose at various concentrations of glucose as described by Wang and coworkers (2004). Control wells containing no cells are included to allow for correction of the conversion of ^3H water into ^3H glucose.

Diaphragm

Glucose utilization by isolated diaphragms can be monitored according to the procedure described by Jeoung and coworkers (2006). For this, diaphragms are removed from 48-h starved mice, rinsed in KHB buffer, blotted, weighed, and placed in 10-ml Erlenmeyer flasks containing 1.5 ml of KHB buffer (pH 7.4), 5 mM glucose, and 0.2% BSA. The flasks are flushed with 95% O_2 /5% CO_2 sealed with rubber stoppers, placed in a shaking (60 cycles/min) water bath at 37°C , and pre-incubated for 30 min. Diaphragms are removed from the flasks, blotted, and transferred to new flasks containing 1.5 ml of KHB buffer supplemented with 5 mM glucose containing 20–500 $\mu\text{Ci}/\text{mmol}$ ^{14}C glucose and 80 $\mu\text{Ci}/\text{mmol}$ ^3H glucose and 1 mU/ml insulin as described by Clark and coworkers (1987). Flasks are flushed with 95% O_2 /5% CO_2 , sealed with rubber serum caps fitted with hanging center wells and incubated for 1 h with shaking at 37°C . Reactions are terminated by the injection of 0.25 ml of phenethylamine/methanol (1/1, by vol.) into the center wells and 0.1 ml of 60% (w/v) perchloric acid into the incubation medium.

EVALUATION

The rate of glucose oxidation is determined from the production of $^{14}\text{CO}_2$, the rate of glycolysis from the difference between the rate of $^3\text{H}_2\text{O}$ formed (Ashcroft et al. 1975) and the rate of substrate cycling, and the rate of substrate cycling from the difference between the rates of glycogen synthesis from ^{14}C glucose and ^3H glucose. Glucose utilization rates are calculated as pmol of glucose utilized/hour/cell or mg tissue as: Glucose utilized (pmol) = ^3H water formed (cpm)/specific radioactivity of ^3H glucose (cpm/pmol).

K.6.2.2**Pyruvate Oxidation****PURPOSE AND RATIONALE**

Pyruvate oxidation can be monitored in isolated rat adipocytes as the incorporation of the acetyl-CoA generated into lipophilic products along the fatty acid syn-

thesis and esterification (into TAG) pathways. Under conditions of glucose deprivation pyruvate dehydrogenase (PDH) is rate-limiting for the overall conversion of 3- ^{14}C pyruvate into radiolabeled products (e. g. fatty acids, TAG) which are separated from the unincorporated ^{14}C -labeled pyruvate, acetyl-CoA and hydrophilic intermediates by partitioning into the toluene phase.

PROCEDURE

Isolated rat adipocytes are prepared by collagenase digestion as described above (K.5.1.2), however, in KRHB lacking glucose. 3.5×10^4 cells are incubated (2 h, 37°C) with 0.1 mM [$3\text{-}^{14}\text{C}$]sodium pyruvate (0.5 μCi) in 1 ml of KRHB lacking glucose. After addition of 5 ml toluene, the samples are vortexed and incubated overnight at 4°C . 1 ml of the upper toluene phase is removed, supplemented with 10 ml toluene-based scintillation cocktail (e. g. Beckman Quickszint 501) and counted for radioactivity.

EVALUATION

Dichloroacetate (DCA), which acts as unspecific inhibitor of PDH kinase leading to activation of PDH and thereby stimulation of pyruvate oxidation, should be used as control (0.2–5 mM final conc.). Data each corrected for a blank value lacking cells are calculated as fold-stimulation of ^{14}C -incorporation into toluene-soluble products in response to DCA vs. basal. In isolated rat adipocytes 5 mM DCA stimulates pyruvate oxidation by 3- to 4-fold. The assay may be useful for the identification of compounds/drug candidates which stimulate pyruvate oxidation by inhibition of PDK (PDK2 in adipocytes) or different modes of action.

K.6.2.3

Pyruvate Dehydrogenase Complex (PDC) Activity

PDC activity is determined according to the method of Jeoung and coworkers (2006). For this, deep-frozen and pulverized diaphragms are homogenized in 5 volumes of extraction buffer containing 30 mM HEPES/KOH (pH 7.5), 0.5 mM thiamine pyrophosphate, 3% Triton X-100, 5 mM EDTA, 2% BSA, 5 mM DTT, 10 μM tosyl-phenylalanyl-chloromethyl ketone, 10 $\mu\text{g}/\text{ml}$ trypsin inhibitor, 1 μM leupeptin, 2 mM dichloroacetate (DCA), and 50 mM KF. The supernatant obtained by centrifugation ($10,000 \times g$, 10 min, 4°C) is made 9% (w/v) in PEG6000 to precipitate PDC. Pellet produced by centrifugation ($12,000 \times g$, 10 min) is suspended in a suspension buffer contain-

ing 30 mM HEPES/KOH (pH 7.5), 1% Triton X-100, 0.2 mM EDTA, 2% BSA, 1 μM leupeptin, 5 mM DTT. For determination of total PDC activity, diaphragms are homogenized in 5 volumes of the extraction buffer lacking DCA and KF. The supernatant (200 μl) obtained by centrifugation of the homogenate ($10,000 \times g$, 10 min, 4°C) is added to 100 μl of an activation buffer (suspension buffer containing 25 mM MgCl_2 , 1.5 mM CaCl_2 , and 1 μg of recombinant pyruvate dehydrogenase phosphatase 1 protein. After incubation (20 min, 30°C) to dephosphorylate and activate PDC, samples are treated with PEG6000 (final 9%, w/v) to precipitate PDC. An aliquot of an extract of the pellet is used to assay of total PDC activity. PDC activity is measured spectrophotometrically in a 96-well plate reader with a coupled assay based on the reaction catalyzed by arylamine acetyltransferase (Nakai et al. 1999, Coore et al. 1971). One unit of PDC activity corresponds to the acetylation of 1 μmol of *p*-(*p*-aminophenylazo)-benzenesulphonate per min at 30°C .

K.6.2.4

Pyruvate Dehydrogenase Kinase (PDK) Activity

GENERAL CONSIDERATIONS

The PDC multienzyme complex (5–10 million Da) catalyzes a key regulatory step in oxidative glycolysis, the irreversible decarboxylation of pyruvate. Its activity is reduced under conditions of increased fatty acid oxidation, i. e. when tissue [acetyl-CoA]/[CoA] and [NADH]/[NAD $^+$] ratios are elevated. Thus, PDC activity is down-regulated during fasting and in pathological conditions associated with insulin resistance, such as diabetes and obesity (Fuller and Randle 1984, Orfali et al. 1993, Kelley et al. 1992).

The mammalian PDC is composed of multiple copies of three enzymes: pyruvate decarboxylase (E1 subunit), dihydrolipoyl acetyltransferase (E2 subunit, a modular protein which contains a transacetylase and so-called outer and inner lipoyl domains, E2 $_c$, E2 $_{L1}$ and E2 $_{L2}$, respectively), and dihydrolipoyl dehydrogenase (E3 subunit). It is regulated by reversible phosphorylation (Linn et al. 1969, Yeaman 1978). Four pyruvate dehydrogenase kinase (PDK) and two pyruvate dehydrogenase phosphatase (PDP) isozymes have been identified to date (Gudi et al. 1995). PDKs inactivate PDC by catalyzing the ATP-dependent phosphorylation of three serine residues on the PDC E1 subunits.

PURPOSE AND RATIONALE

The blood glucose lowering effect of dichloroacetate (DCA) in diabetic rodents (Lorini and Ciman 1962) and type II diabetic patients (Stacpoole et al. 1978)

has been ascribed to its ability to inhibit PDKs and thereby activate PDC *in vivo*. The primary structures of PDK isozymes 1–4 share little sequence homology with other eukaryotic protein kinases and are more homologous to prokaryotic histidine kinases (Popov et al. 1993). These findings increased the attractiveness for drug discovery projects to identify novel inhibitors of PDK activity as possible therapeutic agents for diabetes and other pathological conditions in which PDC activity is reduced (e.g. lactic acidosis, ischemia) (Bersin and Stacpoole 1997). Until recently, the only known small molecule inhibitors of PDK activity were pyruvate, DCA, halogenated acetophenones and analogs of ATP (e.g. adenosine 5'-[β,γ -imido]triphosphate (p[NH]ppA). The following assays may be useful for the identification of structurally novel PDK inhibitors.

PROCEDURE

Functional PDK Assay

Compounds are assessed for their ability to inhibit the ATP-dependent inactivation of porcine heart PDC. The assay essentially consisted of three steps. First, a BSA-stabilized PDC preparation is acetylated to enhance its intrinsic PDK activity. Second, the PDK reaction is initiated by the addition of ATP. Third, after 7 min the PDK reaction is terminated and the residual PDC activity is assessed by monitoring the formation of NADH at 340 nm. In some experiments, the PDC is either supplemented with rPDK and/or ATP is omitted during the second step.

E1 Subunit Phosphorylation Assay

For phosphorylation of the PDC E1 subunit by endogenous PDK (Jackson et al. 1998), the reaction is initiated by the addition of [γ - ^{33}P]ATP (80 mCi/mmol) to intact porcine PDC. The reaction is terminated after 45 s by the addition of TCA. Precipitated protein is recovered by centrifugation, dissolved in 1 M NaOH and the radioactivity of the entire sample is determined by liquid scintillation counting.

PDK Peptide Phosphorylation Assay

PDK activity is determined by measuring the PDK-catalyzed phosphorylation of an acetylated tetradecapeptide substrate, Ac-YHGHSMSPGVSYR, as previously described (Jackson et al. 1998), except that the reaction volume is 25 μl . Effects of compounds/drug candidates on PDK activity are usually determined in the presence of 0.2 mM [γ - ^{33}P]ATP (0.05 $\mu\text{Ci/nmol}$ ATP) and 0.5 mM peptide substrate. In experiments where the [γ - ^{33}P]ATP

(0.05–0.85 $\mu\text{Ci/nmol}$ ATP) concentration is varied, the final peptide concentration is 0.5 mM. When the peptide concentration is varied, the final [γ - ^{33}P]ATP concentration is adjusted to 0.2 mM. Results are expressed as specific activity (nmol of phosphate transferred per mg of PDK protein per 30 min).

Kinase Autophosphorylation Assay

PDK1 or PDK2 (50 $\mu\text{g/ml}$) is incubated in a 25- μl reaction mixture containing MOPS (40 mM, pH 7.2), KH_2PO_4 (20 mM), EDTA (0.5 mM), MgCl_2 (1.8 mM), KCl (30 mM), DTT (2 mM), NaF (10 mM), [γ - ^{32}P]ATP (0.2 mM, 0.01 mCi/mmol) and compounds/drug candidates at 37°C for up to 5 min. The reaction is terminated by the addition of 0.1 volume of a mixture of 425 mM H_3PO_4 and 5 mM p[NH]ppA. The sample is then adjusted to pH 7.4 by the addition of 125 μl of a mixture of 60 mM Tris (pH 8.7) and NaCl (500 mM). The entire sample is then applied to a nitrocellulose membrane and rinsed with TBS (20 mM Tris, 500 mM NaCl, pH 7.5) using a Dot-slot blotting apparatus. Phosphorylated proteins are visualized using a phosphor screen and a phosphorimager (e.g. Storm 840).

Phosphorylation of E1 Protein by PDKs

PDK1 or PDK2 (7.5 μg) is incubated in the presence or absence of 95 μg of acetylated PDC without BSA (Jackson et al. 1998) in a 75- μl reaction mixture containing MOPS (40 mM, pH 7.2), KH_2PO_4 (20 mM), EDTA (0.5 mM), MgCl_2 (1.8 mM), KCl (30 mM), DTT (2 mM), NaF (10 mM) and [γ - ^{32}P]ATP (0.2 mM, 0.01 mCi/mmol). Following a 45-min incubation at 37°C, the reaction is terminated by the addition of an equal amount of Laemmli sample buffer. 40- μl portions are subjected to SDS/PAGE.

Assay for Direct Inhibition by Supplemental PDKs

PDK2 is added to the functional PDK assay described above, which is performed in the absence and presence of ATP. Alternatively, a PDC preparation which is neither stabilized with BSA nor pre-acetylated is incubated (14 $\mu\text{g/ml}$) in 180 μl of 40 mM MOPS (pH 7.4), 0.36 mM EDTA, 30 mM KCl, 1.5 mM MgCl_2 , 120 mM ADP, 2 mM DTT, 10 mM NaF and the additions indicated, at 37°C for up to 10 min. During this step, ADP is omitted in some experiments. The PDC reaction is initiated by the addition of 20 μl of 40 mM MOPS (pH 7.4), 0.36 mM EDTA, 1.5 mM MgCl_2 , 1 mM DTT, 1.1 mM CoASH, 2.2 mM NAD^+ , 2.2 mM pyruvate and 2.2 mM thiamine pyrophosphate. NADH formation over 5 min is monitored at 340 nm.

EVALUATION

The functional PDK assay has a spectrophotometric read-out and measures the effect of PDK activity on PDC function. However the PDK isozyme composition and the stoichiometry of PDK relative to PDC are unknown. Obviously this assay is more difficult to optimize and validate than a traditional kinase assay based upon a model fluorescent peptide substrate. However, the functional PDK assay should identify not only compounds acting at known or presumed sites on the kinase (ATP, lipoamide, or pyruvate binding sites) but also those interfering with interactions between PDK and other PDC protein components (e. g. E1 subunit or the inner lipoyl domain (E2L2) of the E2 subunit of PDC). Although inhibitors of protein–protein interactions are rare, it is desirable that an initial screening assay allows for this possibility.

In contrast to the functional PDK assay, the E1 phosphorylation assay enables PDK activity to be measured directly by testing the compounds/drug candidates for a more specific effect. The ability to inhibit phosphorylation of the E1 subunit by PDK activity intrinsic to the PDC preparation. This assay was used to confirm PDK inhibition in the functional assay. At least one of the concentrations selected for each compound approximated its IC₅₀ value determined in the functional assay. The recombinant inner lipoyl domain of the PDC dihydrolipoyl acetyltransferase (rE2L2) is a known inhibitor of PDK activity in this assay and is included as a control (Jackson et al. 1998).

The E1 phosphorylation assay is able confirm that apparent PDK inhibition in the functional assay is due to inhibition of E1 phosphorylation. However, this assay suffers from the drawback that the PDK isozyme composition and stoichiometry are undefined. Therefore, a much simpler assay with defined stoichiometry of a synthesized tetradecapeptide (a fragment of the E1 subunit which contains all three of the serines which are phosphorylated by PDK [Yeaman et al. 1978]) can be used. This assay has been modified by Mullinax and coworkers (1985) who demonstrated that this tetradecapeptide is a specific substrate for PDK and the PDH phosphatase. Therefore, this assay is in principle the most amenable to kinetic characterization of inhibitors acting at the active site of PDKs.

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K.6.2.5**Fatty Acid Oxidation****GENERAL CONSIDERATIONS**

The development of obesity and the associated insulin resistance results from increases in the ratio of food in-

take to energy expenditure. Loss of body weight can be achieved through a reduction in food intake, a stimulation of energy expenditure, or a combination of both. At the same time, a reduction of fatty acid oxidation has been described in obese and diabetic patients, and the resulting accumulation of fatty acid oxidation has been described in obese and diabetic patients and the resulting accumulation of fatty acids or metabolites of fatty acids in tissues such as skeletal muscle and liver seems to play a direct role in the development of insulin resistance. Recent observations in animal models suggest that a stimulation of fatty acid oxidation reduces the accumulation of fatty acids in tissues and increases overall energy expenditure, leading to lean, insulin-sensitive phenotypes. The modulation of fatty acid oxidation is therefore an attractive new therapeutic strategy for the treatment of obesity and insulin resistance.

Fatty acid oxidation modulated by compounds/drug candidates can be measured with myocytes and hepatocytes by indirect (generation of $^3\text{H}_2\text{O}$) or direct (generation of $^{14}\text{CO}_2$) procedures with the advantages of lower expenditure in equipment and handling inherent to the former and higher accuracy and physiological relevance associated with the latter. In any case current assays using cultured cells are cumbersome and of low-throughput. Most published procedures utilizing ^{14}C -labeled long-chain fatty acid substrates (e. g. palmitate or oleate) are performed in tissue culture flasks, in which a center well captures the radioactive CO_2 product (Angelini et al. 1980, Alam and Saggerson 1998, Kaushik et al. 2001). Alternatively, ^3H -labeled fatty acids can be used, but a labor-intensive extraction of $^3\text{H}_2\text{O}$ is needed (Ibrahimi et al. 1999). These low-throughput assay methods are not suitable for rapid screening of potential drug candidates. More recently, a variant of the $^{14}\text{CO}_2$ -capturing method has been introduced by Collins and coworkers (1998) which facilitates the accurate, reproducible and safe throughput measurement of the released of $^{14}\text{CO}_2$.

K.6.2.5.1 CO_2 Release

PURPOSE AND RATIONALE

The measurement of fatty acid oxidation using a self-contained 48-well assay has been described by Wang and coworkers (2004). Palmitate is conjugated with essentially fatty acid-free BSA to generate a stock solution of 25% (w/v) BSA and 6 mM fatty acid in serum-free medium. After conjugation with albumin, the concentration of fatty acids in the solution is measured

using a NEFA kit (Wako Chemicals, Inc.). The stock solution is diluted into the final culture medium to obtain concentrations of 1 to 1000 μM fatty acid. Palmitate oxidation is measured by the production of $^{14}\text{CO}_2$ from [1- ^{14}C]palmitic acid.

PROCEDURE

For throughput analysis, the cells are incubated for 1 to 3 h at 37°C in 6- to 24-well culture dishes with medium containing 0.2–1 $\mu\text{Ci/ml}$ [1- ^{14}C]palmitic acid and nonlabeled palmitate (1–1000 μM), 2.5 mM glucose and 0.8 mM carnitine in the presence or absence of compounds/drug candidates. Each culture dish is sealed using a special technique, for instance by covering with Parafilm, which had a piece of Whatman paper taped facing the inside of the Petri dish. Alternatively and for low-throughput application, the cells can be handled according to the protocol published by Fedic and coworkers (2006). For this, the L6 myotubes are serum-starved for 4 h before exposure to fatty acids and then incubated for 1 h in 60 \times 15 mm culture dishes with medium containing 0.2 $\mu\text{Ci/ml}$ [1- ^{14}C]palmitic acid (1–10 mM) in the absence or presence of compounds/drug candidates. Each culture dish is sealed with Parafilm, which has a piece of Whatman paper taped facing the inside of the Petri dish. After the incubation, CO_2 generated during this period has to be trapped using a special technique, for instance by wetting the Whatman paper with 100 μl of phenylethylamine/methanol (1/1) and subsequent addition of 200 μl of H_2SO_4 (4 M) to the cells. After additional incubation (1 h, 37°C), the amount of $^{14}\text{CO}_2$ is determined by careful removal of the Whatman paper and transfer to scintillation vials for radioactivity counting as described by Ceddia and coworkers (2000, 2004).

Alternatively, cultured hepatocytes or myocytes are grown to confluence in the odd-numbered rows of 48-well plates. For standard assays, the cells are washed with PBS twice and then incubated with 125 μl of KRB buffer supplemented with 5 mM glucose at 37°C for 1 h. After removal of this buffer, 125 μl of reaction mixture in KRB buffer containing 0.2 μCi [1- ^{14}C]palmitate (\sim 500,000 cpm) and 0.5% BSA is added to the wells. Palmitate is conjugated with essentially fatty acid-free BSA to generate a stock solution of 25% (w/v) BSA and 6 mM fatty acid in serum-free medium. After conjugation with BSA, the concentration of fatty acids in the solution is measured using a NEFA kit (Wako Chemicals). The reactions are performed in the sealed environment of the device at room temperature for 3 h. The reactions are termi-

nated by injection of 250 μ l of 1 M HCl into the cell-containing wells to release CO₂. This injection is performed through the pierceable septum using a syringe. The CO₂ is captured in 250 μ l of 1 M NaOH in the adjacent connected well as room temperature overnight. The NaOH solution is removed into a scintillation vial and mixed with 4 ml of OptiPhase (HiSafe 3) scintillation cocktail and counted.

For measurement of fatty acid in isolated hepatocytes in suspension according to the procedure of Chang and coworkers (2006), cells (5×10^5) are incubated for 1 h in buffer containing carnitine, NAD, ATP, cytochrome c, MgCl₂, coenzyme A, and [¹⁴C]palmitic acid complexed with fatty acid-free BSA. The reaction is stopped with 60% perchloric acid. The released CO₂ is trapped by hyamine hydroxide at the top of a hanging center well and counted for radioactivity in a β -scintillation counter. Fatty acid oxidation is calculated as percentage of the maximal value obtained by stimulation of the cells with epinephrine (1 μ g/ml).

For measurement of fatty oxidation in native liver tissues according to the procedure introduced by Wu and coworkers (2006), liver slices (0.5 mm thick) are prepared using a tissue slicer and placed in 25-ml Erlenmeyer center-well flasks with 2 ml KRP buffer. [¹⁴C]oleic acid bound to fatty acid-free BSA in KRP buffer is added to a final concentration of 0.5 mM and equilibrated for 30 s with a humidified 95% O₂/5% CO₂ gas mix. Flasks are then capped with a rubber stopper containing a centered well enclosing a loosely folded filter paper moistened with 0.2 ml of 1 N NaOH solution. After incubation for 4 h at 37°C, the reaction is stopped by injecting 0.2 ml of H₂SO₄ (1 M). The radioactivity trapped in the filter paper is determined by scintillation counting.

To improve the throughput, a device that accommodates four 48-well culture dishes in a single unit has been designed (Collins et al. 1998). Two adjacent wells form a single experimental unit by means of a connecting chamber, which is sealed from other experimental units and from the outside environment to prevent the release of radioactive material. In this design, one well of the experimental unit contains the cells and ¹⁴C-labeled fatty acid. The [¹⁴C]CO₂ produced by β -oxidation of the fatty acids is then released from the culture medium upon addition of HCl and is subsequently captured in the adjacent NaOH-containing well. The data published demonstrate that the use of this device, which has a simple design and is easy to use, allows highly reproducible evaluation of long-chain fatty acid oxidation in cultured cells with low variability, can be used with a variety of substrates,

such as long- and medium-chain fatty acids, and cell lines, such as HepG2, primary rat hepatocytes and HEK293, and unlike previously reported center-well capture methods for β -oxidation assays, can be run in medium-throughput making it suitable for compound characterization and drug discovery. The ability to use primary hepatocytes opens the possibility to evaluate cells after exposure to drugs *in vivo*.

K.6.2.5.2

Release of Acid-Soluble Metabolites (ASM)

PROCEDURE

Determination of fatty acid oxidation as the release of ASM, which predominantly consist of tritiated water and ketone bodies, follows the procedure described by Moon and Rhead (1987) with modifications introduced by Minnich and coworkers (2001). The reaction mixture is prepared by complete evaporation of the solvent from [9,10-³H]palmitic acid with a 5% CO₂/95% air stream. The [³H]palmitate is resuspended in Hanks' basic salt solution (HBSS) containing 10 mg/ml BSA and brought to a final concentration of 22 μ M [³H]palmitate and 0.5 mg/ml BSA with HBSS. 0.2 ml of this mixture is applied to the wells of 24-well culture dishes containing HepG2 cells or L6 myotubes rinsed twice with 1.5 ml of Dulbecco's PBS. Blanks are prepared by applying 0.1 ml methanol for 30 s. After incubation (2 h, 37°C) in humidified 5% CO₂/95% air, the reaction mixture is removed and added to a centrifuge tube containing 0.2 ml of 10% TCA. Each well is rinsed with 0.1 ml of Dulbecco's PBS, which is added to the tube. After 2 min at room temperature, the reaction mixture is centrifuged (8,500 \times g, 5 min). The supernatants are immediately removed, then supplemented with 70 μ l of 6 N NaOH and loaded onto a 1-ml Dowex-1 column in a pasteur pipette. Columns are rinsed with 1 ml of distilled water. The eluate is collected in a scintillation vial containing 10 ml of scintillation cocktail and then counted for radioactivity.

Alternatively, for bypassing the removal of non-oxidized fatty acids by column adsorption, the ASM can be measured directly by transfer of the cells to plastic tubes according to the protocol introduced by Aas and coworkers (2004). The flasks are rinsed with 1.5 ml of perchloric acid (1 M) that is subsequently added to the same plastic tube. The tubes are then centrifuged (1,800 \times g, 10 min) and 1.0 ml of the supernatant is counted by liquid scintillation. ASM are also measured in the growth media of cells grown on 6-well plates that had been incubated with [1-¹⁴C]oleic

acid and [1-¹⁴C]palmitic acid. A 250- μ l aliquot of the cell medium is precipitated with 100 μ l of 6% BSA and 1.0 ml of 1 M perchloric acid. After centrifugation (1,800 \times g, 2 min), 500 μ l of the supernatant is counted by liquid scintillation. No-cell controls have to be included.

EVALUATION

The sensitivity of this assay critically depends on the substrate concentration, cell density, BSA concentration and incubation time. Though the concentration of [³H]palmitate employed routinely (22 μ M) is not fully saturating, less than 2% of the substrate is converted to ³H₂O by L6 myotubes. The [³H]palmitate concentration is thus virtually constant throughout the incubation period. ³H₂O formation is linear to up to \sim 60 μ g of cell protein per well, at which point the response levels off, and to up to 4 h of incubation. Optimal activity is typically observed in HBSS containing 0.5 mg/ml fatty acid-free BSA in comparison to other media (e. g. MEM). In conclusion, the ³H₂O release assay combines the advantages of relatively low requirements of cells, radiolabeled substrate and assay time.

ASM, a measure of ketone bodies in the liver and of tricarboxylic-acid-cycle intermediates and acetyl esters in muscle, are assayed in supernatants of the acid precipitate. In myocytes, ASM routinely accounts for over 90% of total oxidation products. Minimal increases in ¹⁴C-radioactivity over background levels occur in media in which myocytes have been incubated, indicating that insignificant amounts (<15% of the counts recovered as CO₂) of ASM are released into the medium.

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K.6.2.6

Carnitine Palmitoyltransferase I (CPTI) Activity

PURPOSE AND RATIONALE

Carnitine palmitoyltransferase I (CPT I) which mediates the uptake of long-chain fatty acids into mitochondria, is the rate-limiting enzyme for mitochondrial fatty acid oxidation in mammalian cells. Pharmacological inhibition of CPT I in course of an increase in its physiological negative regulator, malonyl-CoA, as consequence of the inhibition of fatty acid synthase by a natural small molecule (C75) has been demonstrated to lead to appetite suppression. This apparent anti-obesity therapeutic action is presumably based on an alteration in the metabolism of neurons in the hypothalamus, where an increase in malonyl-CoA serves as a secondary messenger of the nutrient status, thereby mediating satiety (Loftus et al. 2000, Hu et al. 2003). In addition, in peripheral tissues (Thupari et al. 2002), C75 has been postulated not only to increase the level of malonyl-CoA but also to act as a malonyl-CoA analog that antagonizes the inhibitory effect on CPT I (McGarry and Brown 1997). Both its central and the peripheral actions could reduce weight in lean and fat mice. Furthermore, both direct activation and direct inhibition of CPT I and the resulting increased

and decreased fatty acid oxidation, respectively, have been implicated as putative target for the improvement of impaired insulin signaling and glucose metabolism in muscle and liver cells in course of reduced lipid accumulation (i. e. reduced lipotoxicity, see K.6.3.2) and of enhanced glucose oxidation according to the inverse relationship between glucose and fatty acid oxidation (i. e. Randle cycle), respectively.

Mammalian tissues express three isoforms of CPT I, liver (L-CPT I), muscle (M-CPT I) (McGarry and Brown, 1997), and brain (CPT I-C) (Price et al. 2002). The liver and muscle isoforms are tightly regulated by their physiological inhibitor malonyl-CoA, which allows CPT I to signal the availability of lipid and carbohydrate fuels to the cell. The malonyl-CoA sensitivity of L-CPT I in the adult rat depends on the physiological state. It is increased by renewed feeding of carbohydrates to fasted rats, by obesity, or following administration of insulin to diabetic rats, whereas it is decreased by starvation and diabetes (Grantham and Zammit 1986, 1988). CPT I activity can be measured with mitochondria from cultured cells and tissues.

PROCEDURE

Preparation of Mitochondrial Fractions

Mitochondria-enriched cell fractions from various cell types (INS, L6 myotubes, HEK293 cells) cultured in 15-cm dishes are obtained with a glass homogenizer as described by Rubi and coworkers (2002). The pellet, in which the mitochondria remain largely intact, is used directly for CPT I activity assays. Mitochondria-enriched fractions are obtained from rat and mouse muscle according to published procedures (Saggerson and Carpenter 1981) with minor modifications. Two soleus muscle samples of each animal are homogenized separately in 250 mM sucrose buffer using an omni-mixer and then centrifuged ($1,000 \times g$, 15 min). The pellet is homogenized and centrifuged ($600 \times g$, 10 min). The resulting supernatant is centrifuged ($15,000 \times g$, 15 min) and the pellet is resuspended in 100 μ l of a buffer containing 250 mM sucrose and 150 mM KCl. Mitochondria-enriched fractions from rat and mouse liver are obtained by homogenization in a buffer containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). The liver suspension is centrifuged ($560 \times g$, 15 min), and the supernatant is further centrifuged ($12,000 \times g$, 20 min). The pellet is resuspended in 2 mL of homogenization buffer, centrifuged ($7,000 \times g$, 10 min), washed and resuspended in 1 ml of the homogenization buffer. To obtain mitochondria-enriched fractions from mice pancreas, tissue is homogenized

in a buffer containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10 μ g/ml leupeptin, 4 μ g/ml aprotinin, 2 μ g/ml pepstatin, and 100 μ M PMSF. The homogenate is subjected to differential centrifugation ($900 \times g$, 10 min and $5,500 \times g$, 10 min). The pellet is resuspended with a Dounce homogenizer and centrifuged ($2,000 \times g$, 2 min and $4,000 \times g$, 8 min). Finally, the pellet is resuspended in 250 μ l of 250 mM sucrose (Li et al. 1996). All the processes are performed at 4°C, and fractions are assayed immediately for CPT I activity.

Determination of CPT I Activity

CPT I activity is measured in mitochondria-enriched fractions obtained from cultured cells or tissues. CPT I activity in 10–15 μ g of mitochondria-enriched cell fractions, or 20 μ g of mitochondrial fractions from tissues is determined by the radiometric method as described by Morillas and coworkers (2001). Extracts are pre-incubated at 30°C for different times in the presence or absence of compounds/drug candidates. Enzyme activity is assayed for 4 min at 30°C in a total volume of 200 μ l. The substrates are 50 μ M palmitoyl-CoA and 400 or 1000 μ M L-[methyl-³H]carnitine for L- and M-CPT I isoforms, respectively. In tissues and cell culture extracts, both CPT I (malonyl-CoA-sensitive) and CPT II (insensitive to malonyl-CoA) are present. Thus, in these fractions, CPT I activity is determined as the malonyl-CoA/etomoxiryl-CoA-sensitive CPT activity. CPT II activity, which is also present in mammalian mitochondrial extracts, is always subtracted from the total activity to calculate specific CPT I activity. The presence of CPT activity insensitive to malonyl-CoA (CPT II activity) in mitochondria obtained from cell cultures is typically less than 5% and thus is not taken into consideration. Compounds/drug candidates are pre-incubated with the enzyme for 1–5 min depending on the assay. Compound/drug candidate concentrations ranging from 0.01 to 50 μ M are used to estimate the IC₅₀ value. Malonyl-CoA (50 μ M) is used for malonyl-CoA inhibition assays. In all cases, the molar ratio of palmitoyl-CoA to BSA is kept at 5:1 to avoid the presence of free acyl-CoA and its deleterious detergent effects and to prevent the formation of micelles. Kinetic constants (K_m and V_{max}) are determined by Lineweaver-Burk analysis. Inhibition constants (K_I and k_{inact}) are determined at 20 μ M palmitoyl-CoA by nonlinear parameter estimation (Maurer and Fung 2000) using SigmaPlot version 8.0. All protein concentrations are determined using the Bio-Rad protein assay with BSA as a standard.

Binding of Compounds/Drugs to CPT I

The binding of compounds/drugs to CPT I is assessed as described by Tutwiler and Ryzlak (1980) with some modifications. Mitochondria-enriched fractions are pre-incubated for 5 min at 30°C with compounds/drugs at 50 μM. One aliquot is used directly for the CPT I activity assay (unwashed), and the other aliquot is centrifuged (13,000 × g, 5 min, 4°C) and resuspended (washed) in 5 mM Tris-HCl (pH 7.2), 150 mM KCl, 2 μg/ml leupeptine, 0.5 μM benzamidine, 1 μg/ml pepstatin, and 1 mM PMSF before the activity assay.

To verify the reversibility of the interaction of compounds/drug candidates with CPT I, dialysis assays are performed. Mitochondria-enriched fractions (160 μg) are pre-incubated at 30°C for 5 min (without the compound/drug) or with a final concentration of 50 μM compound and then dialyzed in buffer containing 10 mM HEPES (pH 7.4), 1 mM EDTA, and 10% glycerol at 4°C. Aliquots are taken before dialysis (0 h), and 24 and 36 h thereafter, and assayed for CPT I activity. This method has been used by Bentebibel and coworkers (2006) to demonstrate novel effects of C75 on CPT I activity and palmitate oxidation.

K.6.2.7**Respiratory Quotient (RQ)**

Respiratory quotient changes during variable periods of fasting of rodents are monitored at 24°C ambient temperature in an open respirometric system. Individual O₂-consumption and CO₂-production are recorded every 5 min at a flow rate of ~40 l/h. RQ equals volumes of CO₂ released/volumes of O₂ consumed. During the 18-h measurement period mice are maintained in metabolic cuvettes (5 l), which contained bedding material (~50 g) and provided access to water *ad libitum*. In each trial, typically 4 mice are investigated. Before and after the experiment, mice are weighed and body temperature are recorded by inserting a rectal probe.

K.6.2.8**Phosphorylation of Acetyl-CoA Carboxylase (ACC) and AMP-Dependent Protein Kinase (AMPK)****PURPOSE AND RATIONALE**

In recent years, a large body of evidence has been published showing that in mammals AMP-activated protein kinase (AMPK) responds to hormonal and nutrient signals in the central nervous system and peripheral tissues, modulating food intake and whole-

body energy homeostasis (for a review see Carling 2005). AMPK is a heterotrimeric enzyme that has been proposed to function as a “fuel gauge” that monitors changes in the energy status of cells. When activated, AMPK shuts down anabolic pathways and promotes catabolism in response to an increase in the AMP/ATP ratio by downregulating the activity of key enzymes of intermediary metabolism. In its activated state, AMPK phosphorylates serine residues 79, 1,200, and 1,215 of acetyl-coenzyme A carboxylase (ACC), producing an 80–90% decrease in the V_{max} of the enzyme, suggesting that AMPK is the physiological ACC kinase. There is also evidence that long-chain fatty acids (LCFAs) act as potent feedback suppressors of lipogenesis by inhibiting ACC activity. ACC is a multifunctional enzyme that, when active (dephosphorylated form), catalyzes the conversion of acetyl-CoA to malonyl-CoA in the *de novo* lipid synthesis pathway. Malonyl-CoA is a potent inhibitor of CPT I) a rate-limiting step for the entry of LCFAs into mitochondria for oxidation (see above). When ACC is inactive (phosphorylated form), a decrease in malonyl-CoA occurs and disinhibits CPT I, thereby increasing the mitochondrial import and oxidation of LCFAs (for reviews see McGarry and Brown 1997, Carling 2005). Therefore, the AMPK/ACC system is thought to play a central role in the regulation of cellular lipid homeostasis (Spiegelman and Flier 2001). In certain metabolic disorders, such as obesity and type II diabetes, lipid metabolism is dysfunctional, causing fatty acids to increase in the circulation and also in intracellular compartments. High levels of fatty acids are toxic to the cells and may cause deleterious metabolic abnormalities (lipotoxicity). By increasing fatty acid oxidation in peripheral tissues, the AMPK/ACC system may play an important role by protecting the cells from these metabolic abnormalities. Of special interest are the mechanisms that regulate the AMPK/ACC system in skeletal muscle, because this tissue plays a major role in determining whole-body energy expenditure, accounts for 70% of total-body glucose disposal, and may modify substrate utilization toward substantially increasing fatty acid oxidation.

The classical view is that AMPK is activated allosterically by an increase in the intracellular AMP/ATP ratio, by phosphorylation of threonine 172 (Thr-172) within the α -subunit, catalyzed by the upstream kinase LKB1 (the upstream kinase of AMPK), and by inhibition of the dephosphorylation of Thr-172 by protein phosphatases. To date, a wide range of physiological stressors, pharmacological agents, and hormones associated with increases in the intracellular

AMP/ATP ratio have been demonstrated to activate AMPK (Corton et al. 1994, for a review see Carling 2005). Fatty acids, another major cellular energy source, may also regulate AMPK activity in skeletal muscle. It has been reported that in perfused rat cardiac muscle, palmitate and oleate significantly increases AMPK activity without causing any significant alteration in AMP/ATP ratio. Another study has reported that exposure to acetate, octanoate, or palmitate causes a significant reduction in AMP/ATP ratio followed by a significant increase in AMPK activity in primary rat hepatocytes (for reviews see Kemp et al. 1999, Ruderman et al. 1999). In contrast to these observations are reports that the AMPK activity of rat liver purified LKB1/STRAD/MOM25 (the upstream kinase complex of AMPK) was inhibited by long-chain acyl-CoA esters *in vitro* (Lizano et al. 2004, Hawley et al. 2003, Boudeau et al. 2003). Consequently, AMPK and ACC represent important targets for future anti-diabetic and anti-obesity therapy *via* activation of fatty acid oxidation in course of activation of the former and inhibition of the latter. The identification of activators of AMPK or inhibitors of ACC, which act directly at the enzyme level or affect the regulation of the enzymes (i. e. induce their phosphorylation) requires reliable cell-based assay systems for measurement of the activity and phosphorylation state of AMPK and ACC in cultured muscle and liver cells and tissues.

Procedure

Cells are grown on six-well plates and incubated for 60 min in the presence or absence of palmitic acid (1–10 μ M). AICAR, a synthetic nucleotide precursor taken up by the cells and phosphorylated to the monophosphate form ZMP, which can accumulate in the cell, mimicks the effect of AMP on AMPK phosphorylation and activation. Therefore, incubation with AICAR (2 mM, 60 min) is used as a positive control for AMPK and ACC phosphorylation in L6 myotubes. Because ACC is a substrate for AMPK, the determination of ACC phosphorylation also serves as an indicator of AMPK activity. Immediately after all treatments, cells are lysed in buffer containing 135 mM NaCl, 1 mM MgCl₂, 2.7 mM KCl, 20 mM Tris/HCl (pH 8.0), 1% Triton X-100, 10% glycerol, and protease and phosphatase inhibitors (0.5 mM Na₃VO₄, 10 mM NaF, 1 μ M leupeptin, 1 μ M pepstatin, 1 μ M okadaic acid, and 0.2 mM PMSF), heated (65°C, 5 min), and passed five times through a 25 gauge syringe. An aliquot of the cell lysates is used to determine the protein concentration in each sample by the Brad-

ford method. Before loading onto SDS-PAGE gels, the samples are diluted 1:1 with 2 \times Laemmli sample buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 50 mM DTT, and 0.01% bromophenol blue). Aliquots of cell lysates containing 30 μ g of protein are subjected to SDS-PAGE (12% and 7.5% resolving gels for P-AMPK and P-ACC, respectively) and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The phosphorylation of AMPK is determined using phospho-AMPK(Thr172) antibody (1:1,000 dilution), which detects AMPK- α only when activated by phosphorylation at Thr-172 (Cell Signaling Technology). ACC phosphorylation is detected using phospho-ACC-specific antibody (1:500 dilution; Upstate Biotechnology), which recognizes ACC when phosphorylated at serine 79 (Ser-79). Equal loading of samples is also confirmed by Coomassie blue staining of all gels.

K.6.2.9 AMPK Activity

Regulating energy levels is a fundamental process in every living organism. At a cellular level ATP must be maintained at relatively high levels (normally about 10-fold above the concentration of ADP) in order to drive essential metabolic processes. At a whole body level, maintaining a balance between energy supply and energy demand is also crucial. Within the Western world the consequences of failing to regulate energy metabolism is ever more obvious as demonstrated by the huge increase in the prevalence of metabolic disorders, such as obesity and type II diabetes (Jonsson 2002). One system that is emerging as a key player in the overall regulation of energy balance is the AMP-activated protein kinase (AMPK) cascade. AMPK is activated in response to ATP depletion (Hardie et al. 1998, Hardie et al. 2003, Carling 2004 and 2005), which causes a concomitant increase in the AMP:ATP ratio (Corton et al. 1994). Once activated AMPK phosphorylates a number of downstream substrates, the overall effect of which is to switch-off ATP utilizing pathways, e. g. fatty acid synthesis, and switch-on ATP-generating pathways e. g. fatty acid oxidation (Hardie et al. 1998, Kemp et al. 1999). In addition to the acute effects of AMPK, activation of AMPK has longer-term effects, altering both gene expression (Foretz et al. 1998) and protein expression (Winder et al. 2000). Although the physiological consequence of these longer-term effects of AMPK are not fully understood, it seems likely that they are involved in the overall regulation of energy metabolism.

AMPK is activated allosterically by AMP and by phosphorylation. AMPK is phosphorylated on T172 which lies within the activation loop of the kinase catalytic domain, a region where many protein kinases require phosphorylation for their activation (Johnson et al. 1996). Phosphorylation of T172 is catalysed by an upstream kinase, the molecular identity of which remained elusive until very recently. Through advances made in the investigation of the regulation by upstream kinases of SNF1, the yeast counterpart of AMPK, the identity of the upstream kinase appears finally to have been revealed. Three closely related yeast kinases, Elm1, Pak1 and Tos3 have been identified that phosphorylate and activate SNF1 *in vitro* (Hong et al. 2003, Sutherland et al. 2003). The amino acid sequences of the catalytic domains of Elm1, Pak1 and Tos3 are most closely related to members of the mammalian Ca²⁺/calmodulin dependent protein kinase kinase (CAMKK) subgroup. Another kinase sharing significant amino acid sequence identity with the three yeast kinases was LKB1 (also known as STK11). Recent studies have shown that LKB1 phosphorylates and activates a number of AMPK-related kinases (Lizcano et al. 2004), and although the physiological role of these kinases is largely unknown, it is possible that some of the tumor suppressor functions of LKB1 are mediated via these kinases rather than AMPK itself. The finding that LKB1 lies upstream of AMPK, however, does provide the intriguing possibility that there may be a link between metabolic regulation and cell proliferation, mediated by AMPK (Carling 2005). The following assays enable the measurement of AMPK activity as a consequence of appropriate phosphorylation/dephosphorylation by upstream kinases or by direct activation/inhibition by compounds/drug candidates which may be helpful for future anti-diabetic and anti-obesity therapy, respectively.

K.6.2.9.1

Measurement with Recombinant AMPK

PURPOSE AND RATIONALE

For analysis of direct effects of compounds/drug candidates on the activity of AMPK, the enzyme has to be purified (e.g. from rat liver, isoform mixture) or heterogeneously expressed (e.g. in *E. coli* or insect cells, specific isoforms) and subjected to kinase assay in the presence of the agents. These test systems may be used for the identification and characterization of activators or inhibitors for AMPK, which both might be helpful for future therapy of diabetes and obesity, respectively.

PROCEDURE

Expression of Recombinant AMPK

A new polycistronic bacterial expression system has been introduced by Neumann and coworkers (2003), which yields milligram amounts of heterotrimeric and functional AMPK. For this, the cDNA sequences encoding the subunits of AMPK (GenBank Accession Nos. X95578, X95577, and U40819) are subcloned by PCR and inserted into individual pET-plasmids (*NdeI/SpeI* sites of pET3-ax, γ 1-subunit, resulting in p γ 1; *NcoI/SpeI* sites of pET-3dx, β 1-subunit, resulting in p β 1; *NcoI/SpeI* sites of pET14dx, α 1-subunit, resulting in pHis- α 1r), harboring an ampicillin resistance gene (*ampR*). Successive restriction and ligation of individual cistrons to the acceptor vector p γ 1 lead to a polycistronic gene consisting of a promoter, ribosome-binding sites (RBS), the sequences of γ -, β -, and His-tagged α -subunits of AMPK, as well as a transcriptional terminator.

Purification of Recombinant AMPK

The tricistronic plasmid p γ 1 β 1His- α 1 is transformed into competent host cells (*E. coli* BL21-CodonPlus (DE3)-RIL) and incubated overnight at 37°C on LB agar containing 150 μ g/ml ampicillin and 25 μ g/ml chloramphenicol. Resuspended cells are used to inoculate LB medium containing appropriate antibiotics. Cultures are grown in a shaker incubator at 37°C. Protein expression is induced with 0.4 mM isopropyl β -D-thiogalactopyranoside final concentration at an O.D. (600 nm) of 0.6 and cultures were grown for additional 4 h. Cells are harvested, washed twice with 0.9% NaCl, resuspended in lysis buffer (15% sucrose, 50 mM sodium phosphate, pH 8.0, 50 mM NaCl, 10 mM imidazole and 1 mM β -mercaptoethanol), and sonicated on ice in a Branson 250 sonifier (50% duty, output 5, 2 min, three times). Insoluble material is removed by centrifugation and the supernatant is loaded onto Ni-NTA agarose (Qiagen). After washing (three column volumes with lysis buffer, 20 mM imidazole) and elution (lysis buffer, containing 250 mM imidazole), the protein is stored at -20°C until use.

Gel filtration Chromatography

For confirmation of complex formation of AMPK holoenzyme, the size of purified AMPK in solution is determined by gel filtration chromatography with a Superose 12 HR 10/30 column (Amersham-Pharmacia) connected to HPLC. Minor non-protein contaminants in Ni²⁺-purified AMPK are removed by batch affinity purification with Blue Sepharose (Amersham-Pharmacia), using elution with 50 mM sodium phos-

phate (pH 8.5), 1 M NaCl, 50 mM AMP, and 1 mM β -mercaptoethanol. One hundred micrograms of protein is then separated in 25 mM sodium phosphate (pH 7.0), 200 mM NaCl, and 1 mM β -mercaptoethanol at a flow rate of 0.75 ml/min and a temperature of 22°C. The column is calibrated for Stokes radii with the following marker proteins: carbonic anhydrase (24.0 Å, 29 kDa), albumin (35.5 Å, 67 kDa), aldolase (48.1 Å, 158 kDa), catalase (52.2 Å, 232 kDa), ferritin (61 Å, 440 kDa), and thyroglobulin (70.0 Å, 669 kDa).

Alternatively, partially purified AMPK holoenzyme from rat liver (up to the blue-Sepharose step according to the protocol of Carling and coworkers [1989] can be used instead of the recombinant version. It is usually obtained in a partially phosphorylated state and therefore does not require the activation step by exogenous upstream kinases, such as LKB1.

Phosphorylation/Activation In Vitro

Ni²⁺-purified recombinant AMPK is activated with a partially purified preparation of AMPKK (purified up to the Q-Sepharose step (Hawley et al. 1996), with the exception that NaF and sodium pyrophosphate are omitted from the buffers) in the presence of 100 μ M ATP, 5 mM MgCl₂, and 1 mM DTT in 50 mM HEPES (pH 7.4), for 30 min at 37°C in a thermostated shaker. Samples are then diluted in HEPES assay buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 10% glycerol, 50 mM NaF, 5 mM sodium pyrophosphate, and 1 mM DTT) and assayed for AMPK activity. For labelling of AMPK with [³²P]ATP, Ni²⁺-purified recombinant AMPK is incubated in the presence or absence of AMPKK (Q-Sepharose pure) in the presence of 100 μ M [γ -³²P]ATP (approx. 400 cpm/pmol), 100 μ M AMP, 5 mM MgCl₂, and 1 mM DTT for 30 min at 37°C in a thermostated shaker. After dilution, AMPK is repurified on NTA–Nickel magnetic agarose beads (Qiagen, Basel) before adding SDS sample buffer and running on 12% acrylamide SDS gels. Radiolabeled AMPK is visualized by autoradiography or phosphorimaging.

Alternatively, for phospho-AMPK (Thr172) detection, the 35%-ammonium sulfate precipitate from treated myotubes or hepatocytes can be used for immunoblotting with polyclonal antibodies to AMPK phosphorylated at Thr172 (Cell Signaling Technology, USA) according to the protocol of Zhou and coworkers (2001).

Radioactive Assay Method

The radioactive AMPK filter assay with [γ -³³P]ATP and a consensus substrate, the so-called SAMS pep-

ptide (HMRSAMSGHLHLVKRR) (Dale et al. 1995) is conducted as described by Zhou and coworkers (2001) and Davies and coworkers (1989). The 100- μ l reaction mixture contained 100 μ M AMP, 100 μ M ATP (0.5 μ Ci ³³P-ATP per reaction), and 50 μ M SAMS in a buffer (40 mM HEPES, pH 7.0, 80 mM NaCl, 0.8 mM EDTA, 5 mM MgCl₂, 0.025% BSA, and 0.8 mM DTT). The reaction is initiated with addition of the enzyme. After 30-minute incubation at 30°C, the reaction is stopped by addition of 80 μ l 1% H₃PO₄. Aliquots (100 μ l) are transferred to 96-well MultiScreen plates (MAPHNOB50; Millipore Corp., USA). The plate is washed three times with 1% H₃PO₄ followed by radioactivity detection in a microtiter plate TopCount apparatus.

AlphaScreen Assay Method

The enzymatic reactions for the Alphascreen are carried out according to Li and coworkers (2003) in white round-bottomed 96-well plates (Packard). Each reaction mixture contained (in a total volume of 0.015 ml) ACC-CREBp (100 nM), ATP (200 μ M), buffer A (50 mM HEPES, pH 7.0, 100 mM NaCl, 0.01% BSA (w/v), 5 mM MgCl₂, 0.8 mM DTTI), and AMPK (0.74 mU, 1 mU = 1 pmol phosphate incorporated into 200 μ M SAMS per min at 30°C in the radioactive filter assay). The reaction is initiated by the addition of the enzyme. Incubation is at 30°C for 30 min. The reaction is terminated by the addition of the detection mixture which contained donor and acceptor beads (40 μ g/ml each), Anti-pS¹³³-CREB antibody (4 nM), and EDTA (16 mM) in buffer A (0.015 ml) without MgCl₂. The plate is read on an ALPHAQuest instrument (Packard Biosignal) after incubation at room temperature in the dark for 2–24 h.

HTRF Assay Method

The enzymatic reactions for HTRF are identical to those for the Alphascreen but are carried out in black round-bottomed 96-well Optiplates (Packard) as described by Li and coworkers (2003). The reaction is terminated by the addition of detection mixture (0.035 ml) which contained SA-XL665 (14 nM), LANCE Eu-W1024-labeled Anti-pS¹³³-CREB antibody (140 pM), and EDTA (3 mM) in buffer A without MgCl₂. The plate is incubated for 1 h at room temperature followed by determination of fluorescence with a Discovery instrument (Packard). The 620- and 665-nm fluorescence signals are counted simultaneously and the ratio (665 nm/620 nm \times 10,000) is recorded for each well.

EVALUATION

Inhibition

The *in vitro* AMPK inhibition data are fit to the following equation for competitive inhibition by non-linear regression using a least-squares Marquardt algorithm in a computer program: $V_i/V_o = (K_m + S)/[S + K_m \times (1 + I/K_i)]$, where V_i is the inhibited velocity, V_o is the initial velocity, S is the substrate (ATP) concentration, K_m is the Michaelis constant for ATP, I is the inhibitor concentration, and K_i is the dissociation constant for the inhibitor. For determination of K_m , AMPK is assayed at varying concentrations of SAMS peptide in the presence of 200 μ M AMP. Kinetic analysis is performed using Graphpad Prism software as has been introduced for AMPK analysis by Stein and coworkers (2000).

Activation

The availability of ultrafast, ultrasensitive, and robust assays suited to high-throughput screening is key to obtaining small-molecule AMPK activators. In the absence of high-affinity and selective anti-phospho Ser/Thr antibodies for AMPK substrates, two homogeneous AMPK assays have been developed with the commercially available antibody Anti-pS¹³³-CREB and an engineered peptide ACC-CREBp. Anti-pS¹³³-CREB antibody was raised against the phospho-CREB peptide derived from cAMP response element binding protein (CREB). ACC-CREBp is a variant (Arg to Pro) of ACC-CREB, a hybrid peptide consisting of a 9-amino-acid peptide from rat ACC, CREB peptide, and the addition of two hydrophobic Leu residues. ACC-CREBp shows increased suitability as a substrate for AMPK, eliminates phosphorylation by PKA, and preserves antibody binding. The homogeneous time-resolved fluorescence and AlphaScreen AMPK assays are developed using both Anti-pS¹³³-CREB antibody and ACC-CREBp that are either labeled with a fluorescent probe or linked to a photoactivated bead, respectively. Very similar biphasic ATP titration curves are generated by both the filter assay and the AlphaScreen. The inhibitory effect observed at higher ATP concentrations most likely results from ATP competing with AMP binding at the allosteric site. Consistent with this hypothesis, an increase in the AMP concentration (from 30 to 200 μ M) shifted the curves to the right, suggesting that more ATP is required to compete with AMP. Nearly identical AMP titration curves are generated by the filter assay and the AlphaScreen.

A small molecule AMPK activator of the thienopyridone class has been identified in a high-throughput screening effort (Anderson et al. 2004, Cool et al.

2006), which directly stimulates partially purified rat liver and recombinant human AMPK ($EC_{50} \sim 1 \mu$ M) and inhibits fatty acid synthesis in primary rat hepatocytes ($IC_{50} \sim 3 \mu$ M) and is lower plasma glucose in ob/ob mice. These results demonstrate that small molecule-mediated activation of AMPK *in vivo* is feasible and represents a promising approach for the treatment of type II diabetes.

K.6.2.9.2

ImmuneComplex Kinase Assay

PURPOSE AND RATIONALE

For analysis of the AMPK activity and its regulation *ex vivo* in (muscle and liver) tissues or cells upon treatment of rodents or cultured cells with putative AMPK modulators, AMPK is immunoprecipitated from total cell or tissue homogenates and then subjected to immune-complex kinase assay using a consensus substrate peptide.

PROCEDURE

Cultured myotubes or hepatocytes, incubated with AICAR or compounds/drug candidates in KRH containing 20 mM glucose for 15 min at 37°C, or alternatively, muscle and liver tissues from rodents, treated with compounds/drug candidates, are homogenized by direct addition of an equivalent volume of ice-cold homogenization buffer (50 mM Tris/HCl, pH 7.4, 250 mM mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na₄P₂O₇, 1 mM PMSF, 1 DTT and 10% glycerol, 0.1% Triton X-100, 1 μ l/ml protease inhibitor cocktail [Sigma]) before snap-freezing in liquid nitrogen. The crude homogenate is sonicated with 4 pulses of 3 s each before centrifugation (18,000 $\times g$, 3 min). Alternatively, Witters and Kemp (1992) lysed treated hepatocytes directly in digitonin-containing and phosphatase-containing buffer, followed by precipitation with ammonium sulfate at 35% saturation.

To 200 μ l of the sample, 2 μ l of α -AMPK antibody (Cell Signaling Technologies) are added, and the immunoprecipitation is incubated overnight at 4°C with gentle mixing. After 12 h immunoprecipitation, 30 μ l of protein-A beads (50% slurry) is added to each sample and incubated for 2 h at 4°C with gentle mixing. The assay is started by the addition of the immunocomplexes to assay buffer (80 mM HEPES, pH 7.4, 160 mM NaCl, 1.6 mM EDTA, 200 μ M SAMS peptide, 200 μ M AMP, 200 μ M ATP, 16% glycerol, 0.1% Triton X-100) containing [γ -³²P]ATP (0.5 μ Ci per sample). After the addition of enzyme to the reaction tube, samples are vortex-mixed for 5 s and in-

cubated for 10 min at 30°C. Following the incubation, the reaction mixture is vortex-mixed and spotted on P81 Whatman filter paper, briefly allowed to dry, and washed three times in 1% HClO₄ before a single wash in acetone. After sufficient time to allow the filter papers to air dry, they are immersed in a scintillant-fluor cocktail and the activity of each sample is measured in a scintillation counter.

K.6.2.9.3

Selectivity vs. Glycogen Phosphorylase (GP) and Fructose 1,6-bis-Phosphatase (FBP)

RATIONALE AND PURPOSE

The enzymes, glycogen phosphorylase (GP) and fructose 1,6-bis-phosphatase (FBP) are allosterically modulated by AMP and ZMP (Longus et al. 2003, Vincent et al. 1991). AMP activates GP, whereas it inhibits FBP. To assess the specificity of small molecules directly activating AMPK, it may be useful to study the effects of the compounds on the activity of GP and FBP. Lack of increase or decrease, respectively, in their activity indicates that unlike AICAR/ZMP these compounds do not act as general AMP mimetics, but selectively activates AMPK. The possibilities of direct interaction with the AMP-binding site of AMPK (but apparently not with those of GP and FBP) or with a distinct allosteric site of AMPK can then be tackled by investigating the putative additive, subadditive or potentiating effects on AMPK activity of combinations of maximally efficacious concentrations of compounds and increasing concentrations of AMP (and *vice versa*). On basis of additive effects obtained by following this strategy between AMP and a newly identified small molecule AMPK activator, which inhibited fatty acid synthesis in primary rat hepatocytes and increases whole-body fatty acid oxidation in normal rats, Cool and coworkers (2006) suggested its binding to a unique site that differs from that of AMP binding.

GP Activity Assay

To assay GP activity according to Kaiser and coworkers (2001), 1.5 µg/ml of rabbit muscle GPb (Sigma) is added to a reaction mix containing 20 mM Na₂HPO₄ (pH 7.2), 2 mM MgSO₄, 1 mM β-NADP (β-nicotinamide adenine dinucleotide phosphate), 1.4 U/ml G-6-PDH (glucose-6-phosphate-dehydrogenase) and 3 U/ml PGM (phosphoglucomutase). AMP or compounds/drug candidates are added to the assay medium followed by the addition of glycogen (final concentration 1 mg/ml) to initiate the reac-

tion. After incubating 10 min at 25°C, GPb activity is assessed by measuring absorbance at 340 nm.

FBPase Activity Assay

FBPase (0.01 U/ml final concentration) in 2× assay buffer (100 mM Tris/HCl, pH 7, 4 mM MgCl₂, 300 mM NaCl, 0.2 mg/ml BSA and 6 mM DTT) is added to the compounds/drug candidates to result in the final desired concentrations. Substrate, D-fructose-1,6-diphosphate, at a final concentration of 0.1 mM is added to the reaction. The reaction mix is incubated at 30°C for 20 min. Following the incubation, 2 volumes of Malachite Green solution (Upstate, USA) containing 0.001% Tween 20 is added and absorbance at 640 nm read immediately.

K.6.2.9.4

AMP:ATP Levels

PURPOSE AND RATIONALE

To confirm that effects elicited by small molecule AMPK activators (e. g. inhibition of fatty acid synthesis, stimulation of fatty acid oxidation) are mediated by direct upregulation of AMPK activity rather than by cytotoxic effects (e. g. uncoupling of mitochondria, blockade of mitochondrial respiration) ultimately leading to decreased levels of ATP and increased levels of AMP, the AMP:ATP ratio of cells can be measured upon challenge with the putative AMPK activator. Lack of effect on AMP:ATP is compatible with the compound exerting its cellular effects by direct activation of AMPK and not as a consequence of cellular stress, toxicity or other means of increasing the AMP:ATP.

PROCEDURE

Primary rat hepatocytes are plated and treated as described above. Following the 4-h incubation with compounds/drug candidates, cells are washed with ice-cold PBS and the adenine nucleotides are extracted by Corton and coworkers (1994) with 500 µl/well HClO₃. The acid is extracted with three treatments of 1.1 volumes of 1/1 tri-n-octylamine:1,1,2-trichlorotrifluoroethane. 50–100 µl of the aqueous sample is injected in a Whatman Partisil 10 SAX column preequilibrated in buffer A (10 mM K₂PO₄, pH 6.75) according to the procedure of Cool and coworkers (2006). For elution, sample columns are run for 5 min in buffer A, then for 15 min in a linear gradient to buffer B (500 mM K₂HPO₄, pH 5) and finally for 35 min in buffer B at a flow rate of 2 ml/min. To measure nucleotide concentration absorbance is read at 254 nm.

K.6.2.9.5**Malonyl-CoA Levels****PURPOSE AND RATIONALE**

Since AMPK activation inhibits ACC activity, one expected *in vivo* consequence is a decrease in cellular malonyl CoA levels, leading to reduced lipid synthesis and elevated fatty acid oxidation (see K.6.2.5). Thus, in conjunction with increased ACC phosphorylation in primary rat hepatocytes, a reduction in malonyl CoA levels may be anticipated in the liver of rats following treatment with compounds/drug candidates.

PROCEDURE

Malonyl CoA levels in rat tissues from animals treated with the appropriate feeding paradigm and sacrificed 1.5 h after treatment with compounds/drug candidates are measured according to the procedure of Cool and coworkers (2006). Tissues are harvested, immediately frozen in liquid nitrogen and then homogenized in a 1:10 volume (w/v) of ice-cold 5% sulfosalicylic acid containing 50 μ M dithioerythritol. Homogenates are centrifuged (15,000 \times g, 60 min, 2°C) and the supernatants filtered through a 0.22- μ m filter (Ultrafree-MC, Millipore USA). Samples are stored at -80°C prior to liquid chromatography mass spectrometry analysis. HPLC is run at binary mode (A: 5 mM dimethylbutylamine and 6 mM HOAc; B: 0.1% formic acid in CH₃CN) at 200 μ l/min with the third pump to deliver post column mixing solvent CH₃CN. The data are acquired on a TOF mass spectrometer using positive ion TOF-MS mode.

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K.6.2.10

Acetyl-CoA Carboxylase (ACC) Activity

GENERAL CONSIDERATIONS

Activation of AMPK increases phosphorylation of various downstream target proteins including ACC, a key component in the regulation and coordination of fatty acid metabolism and catabolism (Carling 2004). ACC, the rate-limiting enzyme for fatty acid biosynthesis, produces malonyl-CoA *via* the catalysis of the carboxylation of acetyl-CoA which acts as inhibitor of CPT1 (Ruderman et al. 2003). Thereby, ACC controls mitochondrial β -oxidation in negative fashion coordinating fatty acid synthesis and degradation in reciprocal fashion. Inhibition of ACC may lead to stimulation of fatty acid oxidation in both muscle and liver with beneficial effects for the therapy of type II diabetes and obesity.

In theory, any component of ACC could be a potential target for drug discovery. In fact, a high-throughput screening assay that screens for inhibitors of the holoenzyme has been described by Soriano and coworkers (2006). However, a more robust assay could increase the potential for finding inhibitors. One assay commonly used to measure CT activity is an enzyme-coupled assay that relies on the absorbance of NADH (Guchhait et al. 1974). High-throughput assay formats based on the UV detection of NADH are problematic because the screening libraries often contain many compounds that absorb strongly at 340 nm, thereby generating false negatives.

K.6.2.10.1

DTNB Method

PURPOSE AND RATIONALE

A modification of the coupled enzyme assay for carboxyltransferase, that avoids the use of NADH de-

tection and therefore offers a potential advantage in a typical HTS environment, has been introduced by Santoro and coworkers (2006). The CT reaction is assayed in the reverse direction in which malonyl-CoA reacts with biocytin (an analog of the biotin carboxyl carrier protein) to form acetyl-CoA and carboxybiotin. The production of acetyl-CoA is coupled to citrate synthase, which produced citrate and coenzyme A. The amount of coenzyme A formed is detected using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB = Ellman's reagent). The assay has been developed for use in both 96- and 384-well microplate formats and validated using a known bisubstrate analog inhibitor of CT. The spectrophotometric readout in the visible absorbance range used in this assay does not generate the number of false negatives associated with frequently used NAD/NADH assay systems that rely on detection of NADH using UV absorbance.

PROCEDURE

The assay buffer consists of 100 mM potassium phosphate (pH 7.6) with 0.1% Tween 20. In preparing the reagent stock solutions, 10 mM malonyl-CoA is dissolved in water, 100 mM OAA is dissolved in assay buffer, 1.2 mM DTNB is dissolved in assay buffer, 100 mM biocytin is sonicated in assay buffer, and ACC is dissolved in assay buffer. Commercially obtained citrate synthase solution is diluted 1:10 in assay buffer. Using the above stock solutions, assay solutions are prepared. "CT solution" contains 30 ml of OAA stock, 3 ml of ACC, and 67 ml of assay buffer, and "CS solution" contains 6 ml of malonyl-CoA stock solution, 36 ml of biocytin stock solution, 6 ml of citrate synthase solution, and 52 ml of assay buffer. Final concentrations of reagents in the assay are as follows: 200 μ M malonyl-CoA, 12 mM biocytin, 10 mM OAA, 200 μ M DTNB, 21.9 nM ACC, and 104 nM citrate synthase.

Automated screening is performed on an integrated core system (e.g. Beckman SAGIAN). The assay plates used are prespotted with 1 μ l of each test compound/drug candidate per well. Using an automated liquid dispenser, 30 μ l of CT solution is added to each assay well. The plates are incubated at room temperature for 5 min 40 s to allow for association of potential slow-binding inhibitors with ACC. An automated liquid handler with 384-tip pod (e.g. Beckman Biomek FX) is used to add 9 μ l of DTNB to each well. The reaction is then initiated by the addition of 20 μ l of CS solution using the liquid handler (with gentle aspiration mixing). The plate is promptly transferred (40 s after the beginning of the CS addition step) to an automated 384-detector (e.g. Molecular Devices

SPECTRAMax Plus), and the absorbance at 412 nm is recorded. A second reading is taken 5 min 40 s later. The HTS assay is carried out at room temperature.

EVALUATION

The quality of this assay for throughput assay for inhibitors to the CT subunit of ACC is demonstrated by a large assay signal window, low data variation among controls, and strong Z' factors. The assay is reliable for many hours and reproducible from day to day. The screening assay described here will be a useful tool in the discovery of potential anti-diabetic and anti-obesity drugs targeting human ACC.

K.6.2.10.2

Phosphate Measurement

Colorimetric Method

All assays are performed using clear 96-well plates (e.g. Costar). The order of addition of assay components depended on the type of experiment performed. For initial velocity measurements, all assay components except the substrate with variable concentration are premixed in 200 mM HEPES/KOH (pH 8). The reaction is initiated by adding an equal volume of the missing substrate. Solutions of varying ATP and acetyl-CoA concentrations are prepared in doubly distilled/deionized water; holo biotin carboxyl carrier protein solutions are prepared in 50 mM HEPES (pH 7.3) and 10% glycerol. Aliquots (50 μ l) are removed from the reaction mix at fixed time points and placed in a 96-well plate preloaded with 100 μ l BioMol Green reagent (BIOMOL) to quench the reaction. Incubation time for color development, typically 30 min, is the same for all mixtures. Absorbance is read at 620 nm using a microplate spectrophotometer. Initial rates are measured within a 10% turnover range of the limiting substrate. Phosphate concentration is determined using a standard curve.

Charcoal Adsorption-Scintillation Counting Method

Alternatively, phosphate can be measured using the charcoal adsorption-scintillation counting method adapted from Stitt and Xu (1988). All assay solutions included a trace of [γ - 33 P]-labeled ATP. Initial velocity measurements are as described for the colorimetric method. At various time points, a 30- μ l aliquot is transferred to an Eppendorf tube, fitted with a 0.45- μ m filter, containing 120 μ l of 5% TCA and 50 μ l of 80 mg/ml activated charcoal. After vigorous mixing, the tube is spun in a microcentrifuge (11,000 \times g, 30 s) to filter out the charcoal. A 50- μ l aliquot of the fil-

trate is added to 7 ml of aqueous scintillation fluid and counted for 1 min in a scintillation counter. To obtain the 100% turnover count (*plus* background), a 30- μ l reaction mix is placed in 170 μ l of 5% TCA without charcoal and a 50- μ l aliquot is counted as above. Background counts, measured by repeating the assay adding water instead of enzyme, are subtracted from all measurements to determine percentage turnover.

Scintillation Proximity Assay (SPA) Method

Assay solutions (33 μ l) containing 5 (or 10) μ M ATP, 1 (or 2) nCi/ μ l [γ - 33 P]-labeled ATP, 20 μ M acetyl-CoA, 40 nM AccC, 40 nM AccA/D, and 80 nM BCCP in buffer A (100 mM HEPES/KOH, pH 8, 150 mM NaCl, 10% glycerol, and 1 mM DTT) are incubated for 1 h. The reaction is quenched by the addition of 10 μ l of freshly made solution A (two volumes of solution B [30 mg/ml wheat germ agglutinin-coated polystyrene imaging beads or 30 mg/ml streptavidin-coated SPA scintillation beads, Amersham-Pharmacia Biosciences, in 50 mM HEPES/KOH, pH 7.3] mixed with one volume of 16 mM ammonium heptamolybdate in 2.4 N HCl solution). Shortly thereafter (but no longer than 40 min after), 10 μ l of 256 mM citrate solution is added. The beads are spun down (2,000–3,000 rpm, 5 min). For experiments using SPA imaging beads, the plate is kept in the dark for at least 2 h before reading the signal using the Leadseeker instrument (Amersham-Pharmacia Biosciences). For experiments using SPA scintillation beads, radioactivity is determined in a TopCount instrument (e. g. Packard) after the plate has been centrifuged.

EVALUATION

Initial velocity measurements as a function of substrate concentration are carried out using either the charcoal adsorption–scintillation counting method, the colorimetric method, or the SPA method. Data are fitted to the Michaelis–Menten equation (using e. g. the GraphPad Prism software).

In general, phosphate determination is a simple and robust method for the measurement of the overall activity of ACC. The monitoring of phosphate production, at low (nanomolar) protein concentrations, is demonstrated to represent the overall activity of ACC due to the coupled activities of biotin carboxylase and CT and the cycling of biotin cofactor attached to BCCP between the two half-reactions. The method has been adapted for a 384-well screening assay format and validated to monitor competitive inhibitors of either biotin carboxylase or CT. The use of holo-BCCP instead of free biotin, as well as the use of physiologically rele-

vant concentrations of the protein components, makes this assay a good model for the *in vivo* ACC system and will facilitate the identification of physiologically relevant inhibitors.

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K.6.2.11

Gluconeogenesis, Ketone Body Formation and TCA Cycle

PURPOSE AND RATIONALE

De novo synthesis of glucose in the liver from precursors such as fructose, pyruvate, lactate, gluconeogenic amino acids, and glycerol is a central mechanism to provide the organism with glucose in times of starvation (Cherrington 1997, Nordlie et al. 1999). On the other hand, when glucose is directly available from external resources, gluconeogenesis is dispensable and consequently needs to be shut off. Integration of these events is complex and occurs through various hormonal and nutritional factors (Barthel et al. 2005). The principal parameters affecting hepatic glucose output are the concentrations of the available gluconeogenic substrates and the activity of a few regulatory enzymes. The activity of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) is regulated by transcriptional and non-transcriptional mechanisms, whereas the third key enzyme, fructose-1,6-bisphosphatase (FBPase), is also regulated through competitive inhibition by fructose 2,6-bisphosphate. Insulin is the most important hormone that inhibits gluconeogenesis. It acts predominantly by suppressing the expression of the genes for the key gluconeogenic enzymes, PEPCK and G-6-Pase (Barthel and Schmolli 2003).

Over the recent years, considerable progress has been made in understanding the mechanisms and molecular details involved in the regulation of hepatic glucose production and its impairment in diabetes. A spectrum of different experimental approaches, ranging from genetic manipulations like knockout models and use of gain or loss of function-mutated constructs in cellular models to the development and application of selective pharmacological compounds, has provided evidence for the convergence of multiple signaling pathways in particular on the level of the transcriptional regulation of PEPCK and G-6-Pase gene expression, the key gluconeogenic genes in liver cells. Analogous to multiple braking systems in cars or motorbikes, the redundancy in the regulation of hepatic glucose production emphasizes the critical importance of this process in the glucose homeostasis of the organism. On the other hand, our increasing knowledge in this field has made it possible to identify a group of promising pharmacological targets, therefore providing a solid basis for the development of new and more potent anti-diabetic drugs.

K.6.2.11.1 Hepatocytes

PURPOSE AND RATIONALE

Isolated hepatocytes can be used to study the effect of drugs on hepatic gluconeogenesis and other hepatic metabolic reactions such as ketone body formation and the tricarboxylic acid cycle.

PROCEDURE

Male Wistar rats weighing 200–300 g are used. The hepatocytes are isolated by perfusion of the liver with collagenase (Berry and Friend 1969, Seglen 1976, Alvarez et al. 1987). The viability of the isolated hepatocytes can be evaluated by the Trypan blue test. Usually 90–95% of the cells exclude the stain and can therefore be regarded as intact. The isolated hepatocytes (20×10^6 cells/vial) are suspended in 3.0 ml of KRB containing 4% BSA. The cell suspension is pre-incubated for 15 min at 37°C in a Dubnoff metabolic shaking incubator gassed with carbogen. The following substrates are added in various combinations, and each sample is incubated for 60 min with 1 mM each of alanine, fructose, glycerol and lactate, and 10 mM pyruvate, or 2 mM sodium palmitate bound to albumin. Compounds/drug candidates are added in concentrations between 0.05 and 5.0 mM. At the end of the 60-min incubation period, 0.2 ml of 70% HClO₄ is added into the medium to stop the re-

action. The reaction mixture is then centrifuged, and the supernatant obtained is used to determine the intermediate metabolites. Glucose is assayed by the glucose-oxidase method, lactate (Gutmann and Wahlefeld 1974), pyruvate (Czok and Lamprecht 1974), acetoacetate (Mellanby and Williamson 1974), and β -hydroxybutyrate (Williamson and Melanby 1974) by appropriate enzymatic methods.

In order to measure the influence on the tricarboxylic acid cycle, (U-¹⁴C) alanine, (1-¹⁴C) glutamate, (1-¹⁴C) pyruvate, (1-¹⁴C) palmitate, or (1-¹⁴C) glucose is added after pre-incubation together with various combinations of compounds and substrates, and incubated for 60 min. The radioactivity is measured by the ¹⁴CO₂-capturing method (Gliemann 1965).

K.6.2.11.2 Perfused Isolated Rat Liver

PURPOSE AND RATIONALE

The technique of liver perfusion has been described extensively by Ross (1972), mentioning in his introductory remarks that the isolated liver was first used by Claude Bernard in 1885. Derived from several modifications, the perfusion of rat liver from the portal vein is the most widely used technique.

EVALUATION

Several parameters can be determined in the effluente, such as net glucose production from lactate and net lactate utilization. The values are plotted against time before and after addition of the compounds and drug candidates, such as insulin or sulfonylureas.

MODIFICATIONS OF THE METHOD

This method has been widely used for studying carbohydrate and lipid intermediary metabolism (Herling et al. 1998) as well as drug metabolism (Milne et al. 1997; 2000; Vuppugalla, 2004). Many variations have been reported predominantly with respect to the animal species used. Chaib et al. (2004) compared isolated perfused livers of rats with those of guinea pigs. Den Butter (1994) used livers from rabbits. Further modifications are related to the direction of perfusion via hepatic artery or portal vein or both simultaneously or in connection with the isolated jointly perfused small intestine (Stumpel et al. (1997 and 2000) as well as the continuous perfusion in a recirculated (see above) or open (non-recirculated) manner (Lopez et al. 1998).

Alexander and coworkers (1992) studied hepatic blood flow regulation in an isolated dual-perfused

rabbit liver preparation. Alexander and coworkers (1995) described a miniaturized perfusion circuit using a novel design of organ bath, to maintain a buoyant preparation, and a high-efficiency miniaturized membrane tubing oxygenator for testing hepatic function during prolonged isolated rat liver perfusion. Kobayashi and coworkers (1991) found inhibition of gluconeogenesis in isolated hepatocytes from normal, fasted rats by the sulfonylurea, glimepiride.

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K.6.2.11.3

Phosphoenolpyruvate Carboxykinase (PEPCK) Activity

PEPCK is measured using the $\text{NaH}^{14}\text{CO}_3$ fixation assay as described by Noce and Utter (1975) and Burcelin and coworkers (1995) with some modifications. 490 μl of rat liver cytosol is added to 500 μl of reaction buffer containing 150 μmol Tris/acetate (pH 7.2), 5 μmol sodium IDP, 10 μmol MnCl_2 , 250 μmol KCl, 10 mM DTT, 2 mM GSH, 400/150 μmol KHCO_3 and 15 μCi $\text{NaH}^{14}\text{C}[\text{O}_3]^-$ (10 μmol , Amersham-Pharmacia). The reaction is started by the addition of 10 μl of 0.4 M phosphoenolpyruvate and terminated after 10 min incubation at 25°C by addition of 1 ml of 6 N HCl and placing the

tube on ice. After dilution with 1 ml of deionized water, unreacted CO_2 ($\text{H}^{14}\text{C}\text{O}_3^-$) is removed by bubbling with N_2 and CO_2 for 30 min each. The reaction mixture is supplemented with 10 ml of aqueous scintillation cocktail (Beckman ReadySafe) and measured for radioactivity by liquid scintillation counting. From each value appropriate blanks are subtracted containing the same ingredients but lacking either cytosol or IDP. Under these conditions and up to the maximal amount of cytosol used, the incorporation rates are linear with both cytosol concentration and time during the first 15 min at least.

K.6.2.12

Glucose Transport

GENERAL CONSIDERATIONS

Skeletal muscle contains several glucose transporters. Evidence indicates that GLUT4 translocation to the cell surface is the major mechanism responsible for regulated glucose uptake in this tissue. Insulin and muscle contractions are the most important stimuli for GLUT4 mobilization from the intracellular storage compartment to the plasma membrane. Nevertheless, other factors like hypoxia, catecholamines and glucocorticoids can alter this mobilization. Contraction and insulin cause increases in the maximal velocity of glucose transport (Nesher et al. 1985) through three mechanisms: (1) a conformational alteration in cell surface glucose transporter leading to increased transport activity, (2) increase in the number of GLUT4 in the plasma membrane, and (3) rapid synthesis of new transporters (Hayashi et al. 1997, Rea and James 1997). The current knowledge on the differential effects of insulin and contraction on glucose transport in skeletal muscle has recently been reviewed by Pereira and Lancha (2004).

PURPOSE AND RATIONALE

Some studies have been developed with the purpose of explaining the two different mechanisms that stimulate glucose transport due to the action of insulin and muscle contraction. One hypothesis is that there are two separate pools of GLUT4 in the skeletal muscle. GLUT4 from one pool is thus translocated by the action of insulin but not of contraction, whereas GLUT4 from another pool is translocated by contraction stimulation, but not by insulin. In any case, on basis of the major contribution of the skeletal muscle in the insulin-stimulated whole-body glucose disposal in humans, compounds/drug candidates which activate the muscle glucose transport by engagement of either the

insulin- or contraction-induced signaling pathway may be helpful for the future anti-diabetic therapy. The following tissue- and cell-based assays can be used for their identification and characterization.

K.6.2.12.1

Method Based on Diaphragms

PROCEDURE

Intact washed rat diaphragms are incubated (30 min, 37°C) in HEPES-buffered saline (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 1 mM MgCl_2 , 5 mM glucose, 0.5 mM sodium pyruvate, 1.5 mM KH_2PO_4 , pH 7.4) under constant bubbling with 95% O_2 /5% CO_2 . The diaphragms are then washed two times with the same buffer lacking glucose and further incubated (30 min) in 5 ml of glucose-free buffer in the presence of insulin/compounds/drug candidates. Glucose transport is initiated by addition of 50 μl of 10 μM 2-[1- ^3H]deoxyglucose (10 $\mu\text{Ci/ml}$) in the absence or presence of 25 μM cytochalasin B (control). After 15 min, the diaphragms are rinsed four times with ice-cold buffer containing 10 mM glucose, 25 μM cytochalasin B, blotted with filter paper and homogenized. Portions of the homogenate are used for protein determination (BCA method).

EVALUATION

1-ml portions of the supernatant of a centrifugation (10,000 $\times g$, 15 min) are mixed with 10 ml scintillation cocktail and counted for radioactivity.

K.6.2.12.2

Method Based on Myocytes

PROCEDURE

Transport studies are carried out using six-well Falcon plates. Cells are routinely plated at a density of 1.5×10^5 cells/well. Medium is aspirated and each well is washed with 10 ml of PBS. Nine hundred microlitres of uptake buffer (PBS containing 1 mg/ml BSA) are added to each well. Transport studies are carried out at 23°C and are initiated by adding 100 μl of 2-[1- ^3H]deoxyglucose (10 $\mu\text{Ci/ml}$) to the desired final concentration (up to 500 μM). At appropriate times, uptake is terminated by rapidly washing the cells twice (less than 15 s) with 10 ml of ice-cold PBS. In the case of 3MG uptake, cells are washed with cold PBS containing 1 mM mercuric chloride. Samples are taken at 15, 30, 45 and 60 s after the addition of radioactive substrate. Cells are solubilized with 1 ml of 0.1% Triton X-100, and 0.8 ml aliquots are counted in 10 ml of

scintillation fluid. Under these conditions, the uptake of 3MG and 2DG is linear with time, and over 95% of the internalized 2DG are phosphorylated. Cells in two wells from each plate are detached with 0.1% trypsin, and counted using a Coulter counter. Studies are carried out in duplicate and each experiment is repeated at least twice. Results are consistent in all cases. Data are analysed by a linear least squares regression fit program (e. g. SlideWrite Plus, version 4.0, Advanced Graphics Software, CA), and by a non-linear regression data analysis program (e. g. Enzfitter program, Biosoft, UK).

EVALUATION

Specific glucose transport (dpm/mg of protein) is calculated as the difference between diaphragm-associated radioactivity measured in the absence (total uptake) and presence of cytochalasin B (non-specific uptake). Under these experimental conditions, transport of 2DG is linear for up to 30 min.

K.6.2.12.3

GLUT4 Translocation in Myocytes

GENERAL CONSIDERATIONS

Understanding the molecular mechanisms of insulin action has been of major interest since the discovery, several decades ago, that insulin stimulates glucose transport *in vivo* by inducing GLUT4 translocation (see above). GLUT4 translocation is a complex vesicular traffic process which includes fusion and docking steps. Herbst and coworkers (1995) showed previously that introduction of a peptide corresponding to tyrosine-phosphorylated IRS-1 motifs into 3T3-L1 adipocytes resulted in a significant increase in the translocation of GLUT4 to the plasma membrane by immunofluorescence staining which mimicked the response to insulin. However, no relative increase in the rate of glucose uptake was observed in those cells. This led to the speculation that some stimulus may bring GLUT4 vesicles to the plasma membrane (docking) but is not sufficient to trigger fusion with the membrane. A converse phenomenon was observed by others upon introducing isoproterenol to rat adipocytes whereby the insulin-dependent gain in glucose transporter detected in isolated plasma membrane was not changed while glucose transport was decreased. It is therefore conceivable that immunostaining of isolated membranes or of permeabilized cells cannot distinguish GLUT4 proteins docked on the plasma membrane from those which are fully fused with it. It is well conceivable that compounds/drug candidates pro-

voke GLUT4 translocation without concomitant glucose transport activation and, *vice versa*, transport activation in the absence of translocation.

PURPOSE AND RATIONALE

Subcellular fractionation, cell photolabeling coupled to immunoprecipitation and immunofluorescence or immunoelectron microscopy have been used to detect translocation of GLUT4 to the cell surface. All of these methods are laborious and suffer from methodological inaccuracies. Subcellular fractionation is cumbersome and produces membranes that are rarely pure. Moreover, quantitative recovery of all membrane compartments is difficult or impossible. Affinity labeling of surface GLUT4/1 followed by immunoprecipitation depends on the ability to obtain quantitative immunoprecipitation and recovery upon SDS-PAGE. This technique results in the incorporation of about 1/10 000 of the label added, thus the signal to noise ratio is low. Moreover, the reactivity of the photolabel can depend on the level of activity of the GLUT in addition to the amount of GLUT exposed at the cell surface. Immunofluorescence detection does not distinguish the native GLUT4 molecules incorporated into the cell membrane from molecules in subplasmalemmal vesicles, and is not a quantitative technique. Immunogold electron microscopy detects antigens at the plasma membrane accurately, but has not been successfully used in a quantitative fashion. None of the above techniques is suitable for large numbers of experiments, as would be required for throughput screening and even characterization of anti-diabetic drugs or of agents interfering with insulin signaling or intracellular trafficking. Therefore, a fast, sensitive and quantitative technique is needed to measure GLUT4 translocation in intact cultured myocytes, which does not rely on immunoprecipitation, SDS-PAGE or use of radioactivity, as required by the photolabeling technique and does not require large amounts of cells or laborious subcellular fractionation.

Previous studies have shown that in CHO cells or other fibroblasts stably expressing heterologous GLUT4, insulin-stimulated translocation of the transfected GLUT4 is not detectable by immunoblot analysis of subcellular fractions, nor by immunofluorescence microscopy (Kanai et al. 1993). However, transfection of GLUT4myc allowed for detection of the exofacial epitope by ¹²⁵I-labeled secondary antibody. By this approach, insulin-induced GLUT4myc translocation was observed in CHO cells (Wang et al. 2000). For study of GLUT4 translocation in L6 myocytes, GLUT4myc has to be introduced into L6 in-

sulin-sensitive muscle cells which express endogenous GLUT4 as well as GLUT1 and GLUT3. The amount of GLUT4myc expressed at the surface of basal cells (5 fmol/mg protein) is usually in the range of that of native GLUT4 (about 12 fmol/mg). This discrete level of expression of GLUT4myc likely allows for its correct localization without saturation of the proteins determining its intracellular sorting.

PROCEDURE

Construction and Culture of GLUT4 Ectopically Expressing L6 Myocytes

For the construction of GLUT4myc-expressing L6 myocytes, the human c-myc epitope (14 amino acids) is inserted into the first ectodomain of GLUT4 as described by Kanai and coworkers (1993). The epitope does not affect GLUT4 activity. GLUT4myc cDNA is subcloned into the mammalian expression vector pCXN (pCXN-GLUT4myc). L6 myocytes are transfected with pCXN-GLUT4myc and pSV2-*bsr*, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride. L6-GLUT4myc myocytes in cell monolayers are maintained in α -MEM supplemented with 10% FBS in a humidified atmosphere containing 10% CO₂/90% air at 37°C. Cells are grown in 12- or 24-well plates for immunofluorescence and on glass coverslips for fixation. Prior to experiments, cells are incubated with serum-free α -MEM supplemented with 25 mM glucose for 5 h.

K.6.2.12.3.1

Method Based on Indirect Immunofluorescence

Quiescent L6-GLUT4myc cells grown on glass coverslips are treated with insulin/compounds/drugs, then rinsed once with PBS, fixed with 3% paraformaldehyde in PBS for 3 min at room temperature, and neutralized with 1% glycine in PBS at 4°C for 10 min. The cells are incubated with PBS containing 10% goat serum and 3% BSA at 4°C for at least 30 min. Primary antibody (anti-c-myc 9E10) is added at a dilution of 1:100 and maintained at 4°C for 30 min. The cells are extensively washed with cold PBS before introducing the secondary antibody (Cy3-IgG, 1:1,000) for 30 min at 4°C. The cells are washed, then mounted and immediately examined by immunofluorescence microscopy.

K.6.2.12.3.2

Method Based on Cell Fixation

PROCEDURE

Quiescent L6-GLUT4myc cells treated with insulin/compounds/drugs are washed once with PBS,

fixed in 3% paraformaldehyde in PBS for 3 min at room temperature. Thereafter, the fixative is immediately neutralized by incubation with 1% glycine in PBS at 4°C for 10 min. The cells are blocked with 10% goat serum and 3% BSA in PBS at 4°C for at least 30 min. Primary antibody (anti-c-myc, 9E10) is then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4°C. The cells are extensively washed with PBS before introducing peroxidase-conjugated rabbit anti-mouse IgG (1:1,000). After 30 min at 4°C, the cells are extensively washed and 1 ml OPD reagent is added to each well. The colorimetric reaction is stopped by addition of 0.25 ml of 3 N HCl for 10 min at room temperature. The supernatant is collected and the optical absorbance is measured at 492 nm. Standard curves are generated using either peroxidase conjugated anti-mouse IgG alone or myc-tag peptide. Myc-tag peptide at various concentrations is coated onto 24-well plates by incubation at 4°C for 24 h then allowed to dry. The plates are rinsed with PBS to remove excess salt and the uncoated spaces are blocked with 10% goat serum and 3% BSA.

EVALUATION

Introduction of a tag c-myc epitope (14 amino acids) into the first exofacial loop of GLUT4 cDNA allows for the direct detection of GLUT4myc molecules translocated to the plasma membrane upon fixation of the intact myocytes under conditions which do not allow access of the anti-myc antibodies to intracellular structures, such as the GLUT4 vesicles. The efficacy of this method has been compared with that of the conventionally used subcellular fractionation and immunofluorescence approaches. Virtually similar effects of insulin on GLUT4 translocation are detected by all three approaches.

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K.6.2.13**Glycogen Synthesis****GENERAL CONSIDERATIONS**

Insulin and contractile activity are major regulators of glycogen metabolism in skeletal muscle (Pederson et al. 2005). Insulin stimulates glycogen synthesis, and postprandially, ~80% of ingested glucose is taken up by skeletal muscle and converted to glycogen (Jue et al. 1989a and b, Shulman et al. 1990). Under these conditions, insulin activates glycogen synthase (GS), as well as glucose transport, *via* translocation of the GLUT4 transporter. Glycogen is a major fuel for the contractile activity of skeletal muscle. During contraction, glycogen is utilized as a source of energy, and it has been demonstrated that, perhaps paradoxically, glycogen resynthesis occurs while glycogen is being broken down (Price et al. 1991, 1994). Presumably, this represents a mechanism for the rapid replenishment of glycogen stores when exercise ceases (Bloch et al. 1994, Ivy and Kuo 1998). The period following exercise is characterized by increased glucose uptake and net glycogen synthesis in skeletal muscle, a scenario similar to insulin stimulation of muscle. Despite the fact that the mechanism of GS activation in response to insulin has been extensively studied, the molecular details of both insulin and contraction-induced activation remain mostly unknown. The following assays enable the demonstration of stimulatory effects on glycogen synthesis in muscle cells of compounds with insulin-like activity which may contribute significantly to their blood glucose-decreasing potency.

K.6.2.13.1**Method Based on Diaphragms****PROCEDURE**

Male Sprague Dawley rats weighing 70–100 g are used. The animals are sacrificed during anesthesia

and the diaphragms are carefully removed, spread out and divided into two equal pieces. The hemidiaphragms are incubated in Krebs-Henseleit buffer gassed with carbogen (95% O₂/5% CO₂) with 5 mM [U-¹⁴C]glucose (0.5 μ Ci/ml) in the presence of insulin/compounds/drugs. After 30 min incubation at 37°C, the hemidiaphragms are blotted on tissue, frozen in liquid nitrogen, and ground in a porcelain mortar and pestle chilled with liquid nitrogen. Samples of the powdered tissue are weighed and dissolved by heating for 45 min at 100°C in 30% KOH (1 ml/100 mg tissue) before ethanol is added to a final concentration of 70%. After 4 h at –20°C, the samples are centrifuged (2,000 \times g, 10 min). The glycogen pellets are washed 3 times with 70% ethanol before the amount of ¹⁴C-labeled glycogen is determined by liquid scintillation counting. Total glycogen is determined according to Lowry and Passoneau (1972) after hydrolysis to glucose (1 N HCl at 100°C for 3 h). The incorporation of [¹⁴C]glucose into radiolabeled glycogen is normalized for the total amount of glycogen.

EVALUATION

The concentration dependence of [U-¹⁴C]glucose uptake and conversion into glycogen by insulin/compounds/drug candidates is determined. The isolated diaphragm of rats or mice is the preferable organ to study the effect of insulin and substances with insulin-like effects, such as sulfonylureas, on muscle tissue or the influence of denervation (Standing and Foy 1970, Smith and Lawrence 1984, Ishizuka et al. 1990, Hothersall et al. 1990).

K.6.2.13.2**Method Based on Myotubes**

L6 myotubes are washed three times with Krebs-Ringer phosphate HEPES (KRPH) buffer (150 mM NaCl, 5 mM KCl, 2.9 mM Na₂HPO₄, 1.25 mM MgSO₄, 1.2 mM CaCl₂, 10 mM HEPES, pH 7.4) supplemented with 0.1% BSA. The cells are then incubated for 30 min to up to 2 h with 25 μ l of reaction medium (glucose-free α -MEM containing 20 mM HEPES, pH 7.4, 0.1% BSA, 100–500 μ M [¹⁴C]glucose [0.1–0.5 μ Ci]) in the presence of increasing concentrations of insulin/compounds/drug candidates at 37°C in a humidified atmosphere of 8.5% CO₂. Subsequently, the reaction medium is removed by aspiration and the cells are washed with 100 μ l of ice-cold PBS. The cells are then disrupted by incubation in 50 μ l of 1 N NaOH for 10 min at 60°C. The cell homogenates are cooled to room temperature and trans-

ferred to 96-well FC/DV filter plates (Millipore) containing 100 μ l of ice-cold ethanol. The plates are then incubated for 2–3 h at 4°C. The precipitate is filtered under vacuum and washed three times with 250 ml of ice-cold 66% ethanol. The filters are dried under a 100-W incandescent lamp. 50 μ l of scintillation cocktail (e. g. Microscint 20, Packard) are added and the wells are sealed. The [¹⁴C]glucose incorporated into cellular glycogen is quantified in a TopCount 96-well liquid scintillation counter.

EVALUATION

In order to identify hypoglycemic drugs with insulin-like or insulin-sensitizing activity, Berger and Hayes (1998) developed an assay for activators of glucose incorporation into glycogen utilizing differentiating L6 muscle cells in 96-well plates. In general, *in vitro* assays of glucose incorporation into glycogen in insulin-sensitive tissue have been used culture media containing the normal physiological concentration, ~5.5 mM, of unlabeled glucose and tracer levels of radiolabeled glucose (e. g. Chou et al. 1987, Robinson et al. 1993). Similarly, using this assay design and L6 myotubes, Berger and Hayes (1998) observed that such conditions do not produce glycogen of a high enough specific activity to be accurately quantified in single-well extracts from a 96-well plate. In contrast, use of glucose-free α -MEM as the reaction media increases the signal by more than one order of magnitude, thereby allowing quantification of single wells in the 96-well format. Consequently, addition of exogenous (non-radioactive) glucose is omitted, which apparently does not lead to physiological alterations in course of the brief incubation with compounds/drug candidates and the subsequent assay period. Glucose incorporation follows in a time and concentration-dependent fashion and is blocked by typical inhibitors of glucose transport. Both insulin and pervanadate exerting insulin-like activity increase glycogen synthesis in concentration-dependent manner. This assay may serve as a high-capacity screen to identify novel compounds that upregulate glucose anabolic metabolism in skeletal muscle.

K.6.2.14

Glycogen Synthase (GS) Activity

GENERAL CONSIDERATIONS

Glycogen metabolism is controlled largely by the coordinated action of the two enzymes glycogen synthase (GS) and glycogen phosphorylase (GP). Both enzymes

are controlled by covalent phosphorylation and by allosteric effectors (Cohen 1986, Roach 1991, Roach et al. 2001). GS undergoes a complex multisite phosphorylation at nine sites by several protein kinases (Roach et al. 2001), most notably PKA, casein kinase I, casein kinase II, GSK-3, and AMPK (Carling and Hardie 1989) which generally lead to inactivation. Important regulatory phosphorylation sites are distributed between the amino- (sites 2 and 2a) and the carboxyl-termini (sites 3a and 3b) of the GS molecule (Lawrence et al. 1983, Skurat et al. 1994, 2000). Full activity can be restored to phosphorylated enzyme by the presence of the allosteric activator glucose-6-phosphate (G-6P). GP is activated by phosphorylation of a single site (GP *a*) by phosphorylase kinase (Cohen 1987). The less active, dephosphorylated form (GP *b*) acquires full activity in the presence of the allosteric effector AMP. Dephosphorylation of all three of these key regulatory proteins, GS, GP, and phosphorylase kinase, is believed to be catalyzed primarily by glycogen-associated phosphatases (PP1G) (Hubbard and Cohen 1989a, b).

K.6.2.14.1

Method Based on Diaphragms

PROCEDURE

GS is assayed according to Oron and Lerner (1979), Guinovart and coworkers (1979), Altan and coworkers (1985) with the following modifications: Intact hemidiaphragms are dissected from male Wistar rats and incubated in DMEM (10 ml/hemidiaphragm) with constant bubbling of 95% O₂/5% CO₂. For treatment with insulin/compounds/drug candidates, the hemidiaphragms are incubated (37°C) in the same medium containing 5 mM glucose. For preparation of homogenates, the diaphragms are blotted and frozen in liquid nitrogen. The frozen diaphragms (pool of four) are manually ground in a porcelain mortar and then homogenized at 0°C in 10 vol. of 25 mM Tris/HCl (pH 7.4), 100 mM NaF, 5 mM EDTA, 0.1 mM PMSF. The homogenate is centrifuged (10,000 \times g, 20 min). The supernatant is used for the GS assay. After addition of 10 μ l of diaphragm homogenate to 200 μ l of 25 mM Tris/HCl (pH 7.4), 50 mM NaF, 10 mM EDTA (pre-incubated at 30°C) containing either 0.1 or 10 mM G6P, the reaction is initiated by supplementing 0.2 mM [U-¹⁴C]UDP-glucose (10 μ Ci) and terminated after 15 min by the addition of 2.5 ml of ice-cold 66% ethanol and filtration over pre-wetted Whatman GF/C glass fiber disks. The filters are washed, dried and counted for radioactivity. Blanks are assayed by

adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol.

EVALUATION

The fractional velocity as parameter for the portion of GS active *in vivo* (l-form) toward the total enzyme contents (l+d-forms) at the time point of homogenization is calculated as ratio between the activities measured at 0.1 (l-form) and 10 mM G-6P (l+d-forms).

The concentration-dependence of sulfonylurea stimulated GS and its potentiation by insulin in the isolated rat diaphragm were studied by Müller and coworkers (1994).

in the presence of 0.1 mM glucose instead of 5 mM glucose (the concentration routinely used) did not reveal significant differences with respect to both the activity ratio and the effect of insulin. Presumably, the dilution of the limited amount of G6P, which accumulates during incubation with 5 mM extracellular glucose, during the subsequent preparation of the homogenate and the GS assay is high enough to prevent allosteric activation of GS. According to our experience incubation of the isolated rat adipocytes in the presence of 5 mM glucose has a positive impact on their viability, in general, and insulin sensitivity (glucose transport, glycogen synthesis), in particular.

K.6.2.14.2

Method Based on Myotubes/Hepatocytes

PROCEDURE

GS activity is assayed by measuring the incorporation of D-[¹⁴C]glucose from UDP-[¹⁴C]glucose into glycogen. L6 myotubes or HepG2 cells cultured in 6- or 12-well plates and serum-deprived overnight are incubated with insulin/compounds/drugs in serum-free α -MEM under an atmosphere of 5% CO₂ for 30 min at 37°C. After removal of the incubation medium and washing, the cells are scraped into 0.2–0.5 ml of 10 mM Tris/HCl (pH 7.5) containing 10 mM EDTA, 150 mM KF, 5 mM DTT. The cell lysates are centrifuged (5,500 \times g, 2 min, 10°C). The infranant below the lipid layer is cleared from residual lipids by two additional centrifugations (defatted homogenate) and served as source for GS. The reaction is started by adding 30 μ l of the homogenate to 60 μ l of a reaction mixture (prewarmed at 30°C) containing 33 mM Tris/HCl (pH 7.8), 0.2 mM UDP-[U-¹⁴C]glucose (4 μ Ci), 6.7 mg glycogen, 150 mM KF and 0.1 mM/10 mM G-6P. After incubation for 20 min at 30°C, the reaction is terminated by addition of 2 ml of 66% ethanol, 10 mM LiBr (–20°C), rapid mixing and filtration over pre-wetted Whatman GF/C glass-fiber discs. The filters are washed 5 times with 5 ml of 66% ethanol each at 25°C, dried and measured for radioactivity. Blank values determined by adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol are subtracted from the total values each.

EVALUATION

The fractional velocity is calculated as the ratio of GS activity in the presence of 0.1 mM and 10 mM G6P. Measurements of GS activity in defatted homogenates from myotubes/hepatocytes which had been incubated

K.6.2.15

Phosphorylation State of GS

PURPOSE AND RATIONALE

A large body of evidence suggests that insulin activation of GS proceeds via the PI-3K/Akt pathway that leads to phosphorylation and inhibition of GSK-3 (Shepherd et al. 1995, Cross et al. 1995). However, GSK-3 alone is not sufficient to account for GS dephosphorylation and activation by insulin (Lawrence and Roach 1997, Skurat et al. 2000). The mTOR, mammalian target for the immunosuppressant drug rapamycin, pathway is also activated by insulin. Rapamycin has been shown to block insulin-mediated activation of GS in muscle and 3T3-L1 adipocytes (Azpiazu et al. 1996, Shepherd et al. 1995) without affecting insulin-induced inactivation of GSK-3 (Cross et al. 1997), opening the possibility that mTOR could control GS phosphorylation *via* a phosphatase. Therefore, insulin may promote glycogen synthesis both *via* inhibition of GSK-3 and stimulation of a glycogen-associated type 1 serine/threonine protein phosphatase, PP1G. Studies with mice deficient in the regulatory subunit of PP1G indicate that although PP1G is not necessary for activation of GS by insulin, it is essential for regulation of glycogen metabolism under basal conditions and in response to contractile activity, and may explain the reduced muscle glycogen content in the KO mice, despite the normal activation of GS (Aschenbach et al. 2001). Contractions may utilize a separate signaling pathway from insulin to activate GS in response to contractions. Changes in GS activity in human muscle biopsy samples obtained during isometric contractions are associated with changes in protein phosphatase activity (Katz and Raz 1995), but the identity of this enzyme has not been determined. The phosphorylation state of GS in muscle and liver cells and the impact of compounds/drug candidates can be

monitored by ^{32}P -labeling and subsequent immunoprecipitation of GS.

PROCEDURE

Myotubes/hepatocytes are washed twice by flotation in low phosphate medium composed of KRP-HEPES modified to contain $50\ \mu\text{M}$ KH_2PO_4 , 2 mM glucose, 2% BSA and then suspended in the same medium (7.5×10^5 cells/ml). 5-ml portions are incubated with [^{32}P]phosphate (0.2 mCi/ml) for 2 h prior to addition of insulin/compounds/drug candidates and of 0.5 ml of 50 mM glucose. After incubation (20 min, 37°C), the cells are floated by centrifugation ($1,000 \times g$, 1 min) and the infranatant is aspirated. The cells are suspended in 1.5 ml of cold 50 mM Tris/HCl (pH 7.6), 100 mM KF, 20 mM glycerol-3-phosphate, 10 mM $\text{K}_4\text{P}_2\text{O}_7$, 10 mM EDTA, 1 mM benzamidine, 0.2 mM PMSF and homogenized by 10 strokes using a tight-fitting Teflon-in-glass homogenizer in the same Eppendorf cup. The homogenate is centrifuged ($18,000 \times g$, 30 min, 4°C). 1 ml of the post-mitochondrial supernatant is incubated with 10 μl of GS antiserum raised in guinea pigs by immunization with purified GS from rabbit skeletal muscle. After incubation for 2 h at 4°C , 50 μl of protein A-Sepharose (10% w/v in TES, see below) is added and the incubation continued overnight. The immunoprecipitates are collected by centrifugation ($12,000 \times g$, 2 min, 4°C) and washed twice with 1 ml each of TES (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl) containing 1% TX-100, twice with TES and finally once with 50 mM Tris/HCl (pH 7.4). The beads are suspended in 25 μl of 2xLaemmli sample buffer, heated (95°C , 5 min) and centrifuged. The supernatant is subjected to SDS-PAGE (8% resolving gel). The amount of [^{32}P] contained in GS is determined by excising the corresponding gel pieces and measuring their radioactivity by liquid scintillation counting.

K.6.2.16

Protein Phosphatase 1G (PP1G) Activity and Phosphorylation

PP-1 Activity

Muscle PP1 activity is measured according to a protocol from Begum and Ragolia (1996). L6 myotubes treated with insulin/compounds/drug candidates are scraped off the 24-well dishes with 0.3 ml of phosphatase extraction buffer containing 20 mM Tris/HCl (pH 7.2), 2 mM EDTA, 2 mM EGTA with 10 $\mu\text{g}/\text{ml}$ each of aprotinin, leupeptin, antipain, soy bean trypsin inhibitor, 1 mM benzamidine, 1 mM PMSF. The cells

are sonicated for 10 sec, centrifuged ($2,000 \times g$, 5 min) and the supernatants are used for the assay of PP1G activity in the presence and absence of 2 nM okadaic acid to inhibit PP2A activity. The conditions of the assay allow measurement of PP1 and PP2A activities and not PP2B and PP2C enzymes, which require divalent ions. Okadaic acid at 2 nM inhibits PP2A, only, and the PP-1 activity remaining in the assay is comparable with the activity inhibited by inhibitor 2 (Srinivasan and Begum 1994). Purified [^{32}P]-labeled GP *b* is used as a substrate. [^{32}P]-labeled GP *b* is prepared by reacting γ [^{32}P]ATP with purified phosphorylase kinase and GP *a* (Cohen 1983).

In Vivo Phosphorylation and Immunoprecipitation of PP1

Differentiated L6 myotubes are serum-starved overnight. The next day, the medium is removed and replaced by 1 ml of phosphate-free DMEM and incubation is continued for 1 h. [^{32}P]-orthophosphate is added (0.5 mCi/ml), and the cells are incubated for 4 h followed by incubation with compound/drug candidate for up to 1 h. Subsequently, the cells were rinsed four times with 1 ml of ice-cold PBS containing phosphatase and protease inhibitors and harvested in 0.5 ml of lysis buffer (20 mM triethanolamine, pH 7.2, 0.5 mM EGTA, 1 mM EDTA, 2 mM sodium vanadate, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 40 mM glycerol-3-phosphate, 1 mM benzamidine, 0.1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ each of leupeptin, aprotinin, antipain, trypsin inhibitor, and pepstatin A, 140 mM NaCl, and 1% Triton X-100). The cell lysates are centrifuged (16,000 rpm, 10 min) to remove cell debris. 100 μg of cell lysate protein are diluted to 1 ml with lysis buffer and precleared by incubation with rat IgG (5 $\mu\text{g}/\text{ml}$, coupled to protein A sepharose) at 4°C for 1 h. The supernatants are immunoprecipitated with PP1G subunit antibody (10 $\mu\text{g}/\text{ml}$) for 1 h at 4°C followed by treatment with 50 μl protein A sepharose CL6B (50%, by vol.). The pellets are washed four times with 1 ml of lysis buffer and resuspended in 40 μl of 2-fold SDS-sample buffer. The samples are incubated (37°C , 10 min) followed by centrifugation ($10,000 \times g$, 1 min) to pellet the sepharose beads. Electrophoresis of the immunoprecipitates is performed in 7.5% SDS-polyacrylamide gels followed by autoradiography. The protein contents of PP1G regulatory subunit are determined by immunoprecipitating unlabeled cell lysates with anti-G subunit antibody, followed by separation of immunoprecipitated proteins on SDS-PAGE. The proteins are transferred to polyvinylidene difluoride membrane and probed with PP1G subunit antibody. The regulatory subunit

of PP1 is identified by incubating with [¹²⁵I]protein A (0.2 μCi/ml) followed by autoradiography. The intensity of the signal is evaluated by densitometric analysis of the autoradiograms as well as by radioactivity scanning.

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- with intramyocellular TAG accumulation (Storlien et al. 1991, Oakes et al. 1997, Griffin et al. 1999, Boden et al. 2001, Perseghin et al. 1999, Chalkley et al. 1998). Weight loss and lowering of plasma free fatty acids reduced insulin resistance and the TAG content (Roden et al. 1996, Boden et al. 2001). Several studies have shown an association between obesity, insulin resistance, and reduced oxidative capacity in skeletal muscle (Bachmann et al. 2001, for reviews see Guo 2001, Saloranta and Groop 1996, Boden 1997), as demonstrated by the finding of decreased mitochondrial number, altered mitochondrial morphology and decreased expression of mitochondrial genes of the oxidative metabolism (Morino et al. 2005, Petersen et al. 2004, Mootha et al. 2003, Pati et al. 2003). Deficiency of CPT leads to pathological TAG accumulation in muscle tissue further emphasizing the role of mitochondria for lipid homeostasis. Our current knowledge on TAG accumulation in relation to insulin resistance and type II diabetes originates mainly from *in vivo* studies rendering it difficult to determine the contribution of genetic and environmental factors to TAG accumulation in type II diabetes and obesity. Cultured myotubes offer a unique model for separating the genetic influence on insulin resistance and type II diabetes from environmental factors. The following assays can be used for the analysis of lipid metabolism in cultured muscle cells and its modulation by compounds and drug candidates.

K.6.2.17**Lipid Metabolism in Muscle and Liver Cells****GENERAL CONSIDERATIONS**

Type II diabetes is characterized by hyperglycemia, hyperinsulinemia, reduced ability to oxidize fat, and TAG accumulation in skeletal muscle fibers. Impaired glucose transport and glycogen synthesis are well documented in insulin-resistant subjects (Richter et al. 1988, Hansen et al. 1992, Boden et al. 1996, Kawanaka et al. 2001), but lipid metabolism is less clearly understood (Goodpaster and Kelley 1998). Especially, focus has been put on the increased intramyocellular TAG content, since an inverse correlation between insulin resistance and intramyocellular TAG has been demonstrated (Pan et al. 1997, Koyama et al. 1997, Simoneau et al. 1995, Phillips et al. 1996, Boesch et al. 1997, Szczepaniak et al. 1999). The mechanism responsible for TAG accumulation remains unclear, but it may depend on either an increased lipid uptake or a decreased lipid oxidation as *de novo* lipogenesis within skeletal muscle is low. Increased plasma-free fatty acid (FFA) availability induces insulin resistance within hours by lipid infusion or within weeks by feeding rats a high-fat diet or in massively obese humans accompanied

K.6.2.17.1**Incubation with Fatty Acids**

Solutions containing fatty acids are prepared according to the procedure described by Montell and coworkers (2002). Sodium salt of oleic acid is prepared immediately prior to utilization by dissolving the fatty acid in deionized water containing 1.2 eq of NaOH at 70°C with stirring until an optically clear dispersion is obtained. The fatty acid-salt solution is immediately added to DMEM containing fatty acid-free BSA with continuous agitation to avoid precipitation. Typically, the fatty acid:BSA molar ratio is 5:1. Myocyte cultures are grown in a DMEM/M199 medium (3/1) supplemented with 10% fetal bovine serum, 10 µg/ml insulin, 2 mM glutamine, 25 ng/ml fibroblast growth factor, 10 ng/ml epidermal growth factor. Immediately after myotube differentiation, cells are rinsed in Hanks' balanced salt solution, and a medium devoid of fibroblast growth factor, epidermal growth factor, and glutamine is added. Myotube cultures are maintained in this medium for up to 2 weeks.

K.6.2.17.2**Lipid Synthesis*****Incorporation of [¹⁴C]Glycerol into Lipids***

For qualitative analysis of esterification of glycerol into AG, cells are incubated with 5 mM [¹⁴C]glycerol (100 μCi/mmol), 5 mM glucose, and 1 mM sodium oleate (BSA:oleate molar ratio of 1:5) for 15 h (Montell et al. 2002). The cell monolayers are then washed three times in Hanks' balanced salt solution, and the lipids are extracted twice with hexane/isopropanol (3/2). After drying under nitrogen, the residual lipid extract is redissolved in chloroform/methanol (2/1) as described (Folch et al. 1957) and separated by TLC by use of hexane/diethyl ether/acetic acid (70/30/1). The lipid spots are identified by iodine vapor and counted in a phosphorimager.

Incorporation of [¹⁴C]Glucose into Lipids

The synthesis of neutral lipids starting from glucose can be followed according to the protocol of Aas and coworkers (2004). Myotubes are incubated with either [¹⁻¹⁴C]oleic acid (18.5 kBq/ml, 0.6 mM) or [¹⁻¹⁴C]palmitic acid (18.5 kBq/ml, 0.6 mM) in the absence or presence of insulin/compound/drug candidate for 4 h before they are harvested into ice-cold PBS, centrifuged (1,000 × g, 5 min), resuspended in distilled water and sonicated. Cell-associated lipids are extracted with chloroform/methanol as described (Folch et al. 1957). Briefly, 400 μl of cell homogenate is mixed with 8 ml of chloroform/methanol (2/1, by vol.), and FCS (30 μl) was added as a carrier. After 30 min, 1.6 ml of 0.9% NaCl (pH 2) is added and the mixture is centrifuged (1,000 × g, 5 min). The organic phase is evaporated under a stream of nitrogen at 40°C. The residual lipid extract is re-dissolved in 200 μl of n-hexane and separated by TLC using hexane/diethylether/acetic acid (65/35/1) as the mobile phase. The bands are visualised with iodine, excised and counted by liquid scintillation. Lipids are also extracted after incubation of myotubes with D-[¹⁴C(U)]glucose (74 and 111 kBq/ml, 5.5 mM or 20 mM) for 24 h.

EVALUATION

Extraction of lipids is carried out after incubation of myotubes with D-[¹⁴C(U)]glucose for 4 h. Myotubes are exposed to DMEM supplemented with 0.24 mM fatty acid-free BSA, 0.5 mM L-carnitine, 20 mM HEPES, 5.5 or 20 mM [D-¹⁴C(U)]glucose [(2.0 μCi/ml), and insulin/compound/drug candidate. On the basis of the typically very low incorporation,

the overall glucose incorporation into lipids is determined by counting the total lipid extract washed with a water phase that contained 0.5 M glucose to displace all glucose labeled.

Incorporation of [¹⁴C]Palmitate into Lipids

For analysis of lipid synthesis starting with fatty acids according to the protocol of Busch and coworkers (2005), cells are treated for 48 h with insulin/compounds/drug candidates. Two hours before the end of the treatment 2.5 μCi [¹⁴C]palmitate in ethanol is added to each well (24-well dish, 0.5 ml culture medium). The labeled cells are put on ice, washed in 3 × 0.5 ml ice-cold PBS, scraped off the dish in 1 ml cold PBS, and spun (3,000 × g, 10 min). The supernatant is discarded, and lipids in the cell pellet are extracted overnight in 1 ml chloroform/methanol (2/1, by vol.). The extractions are washed in 250 μl H₂O, followed by an additional wash in 125 μl H₂O, and the resulting lipid-containing, organic phase is dried under a stream of nitrogen. The lipids are re-dissolved in 50 μl chloroform/methanol (2/1, by vol.), and a small fraction is used to determine the total amount of counts in the extracted lipids. The rest of the sample is spotted onto silica plates. The lipids are separated by TLC-D-¹⁴C(U) in petroleum ether/diethyl ether/methanol/acetic acid (180/14/4/1, by vol.). Spots comigrating with TAG and cholesterol ester standards are individually scraped and counted by liquid scintillation spectrometry. Protein is measured in parallel using a bicinchoninic acid protein assay.

Incorporation of [¹⁴C]Acetate into Lipids

This protocol has been adapted from methods previously described (Foretz et al. 1998, Garcia-Villafranca et al. 2003, Cool et al. 2006). Primary rat hepatocytes are isolated as described (see K.5.2.2) and plated at 5 × 10⁴ cells per well on collagen-coated, black-walled 96-well plates in DMEM supplemented with 10% FBS, 5 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES/KOH (pH 7.4), 0.1 mM non-essential amino acids, 5 μg/ml transferrin, 100 nM dexamethasone, 100 nM insulin and 25 μg/ml gentamycin. After 4 h incubation, medium is replaced with medium as described above but lacking FBS and containing 100 nM triiodothyronine. Following a 16 h incubation at 37°C, the medium is removed and replaced with medium containing [¹⁴C]acetate (2 μCi/ml) and as a control, AICAR or compounds/drug candidates. Cells are incubated (4 h, 37°C). Subsequently, the plates are rinsed with PBS three times. The final wash is replaced with scintillation cocktail (e. g. Mi-

crosscint20, Perkin Elmer) and radioactivity incorporated into fatty acid monitored on a microplate β -reader.

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K.6.2.17.3

Lipolysis

GENERAL CONSIDERATIONS

TAG is accumulated in LD in the cytoplasm of skeletal muscle cells (Schick et al. 1993, Oscai et al. 1990). The energy content of this TAG store is higher than the energy content of the muscle glycogen pool. The

muscle TAG concentration is increased by a high-fat diet (Van der Vusse et al. 1996) and in poorly controlled diabetes (Standl et al. 1980, Stearns et al. 1979), and is inversely related to whole-body insulin action (Pan et al. 1997). On the other hand, although existing studies are not unambiguous, the general view is that the intramuscular TAG stores can be mobilized by catecholamines (Froberg et al. 1975, Abumrad et al. 1980) and by exercise (Oscai et al. 1990, Van der Vusse et al. 1996). The exercise-induced decrease in muscle TAG concentration can be reduced by β -adrenergic blockade, and must accordingly be due to some extent to sympathetic stimulation (Stankiewicz-Choroszuca and Gorski 1978). While it seems that the intramuscular TAG constitute a dynamic energy store, the enzymic regulation of TAG breakdown in muscle is poorly understood (Van der Vusse et al. 1996, Martin 1996, Hagstrom-Toft et al. 1997, Kerckhoffs et al. 1998). The expression of HSL and its regulation by adrenaline has been investigated by Langfort and coworkers (1999).

It is thought that HSL is the major enzyme responsible for the hydrolysis of stored TAG in skeletal muscle similar to in adipose tissue. HSL protein or mRNA has been detected in rodent (Holm et al. 1987, Langfort et al. 1999) and in human (Roepstorff et al. 2004) skeletal muscle but with a considerable lower expression than in adipose tissue. The HSL protein expression also varies between fiber types, being higher in oxidative than glycolytic fibers (Langfort et al. 1999). Furthermore, it was recently shown (Hämmerle et al. 2002) that HSL-deficient mice accumulated DAG in adipose tissue and skeletal muscle, indicating that when HSL is missing this leads to an incomplete hydrolysis of TAG with an interruption of the lipolytic cascade at the stage of diacylglycerol hydrolysis. This observation was supported by the findings of a marked reduction in the formation of fatty acids in skeletal muscle as in several other tissues (Hämmerle et al. 2002). Interestingly, when the specific TAG hydrolase activity (the enzymatic conversion of TAG to DAG) was calculated, the specific TAG hydrolase activity was reduced 50% in adipose tissue, whereas no reduction was found in skeletal muscle when comparing HSL-deficient mice with wild type (Hämmerle et al. 2002). These data suggest that HSL is rate-limiting in the catabolism of DAG but not of TAG hydrolysis in skeletal muscle and furthermore points to the existence of one or more lipases with considerable activity, specifically to TAG hydrolysis in skeletal muscle. Further support for the existence of lipases other than HSL involved in basal TAG hydrolysis in skeletal muscle appears from recent

studies, where neutral lipase activity in human skeletal muscle only decreased by $\sim 25\%$ when antiserum against HSL was added to the assay medium (Roepstorff et al. 2004, Watt et al. 2004). When a similar approach was taken in basal, resting rat soleus muscle (Langfort et al. 2000, Langfort et al. 1999), HSL activity was decreased by $\sim 60\%$, indicating that HSL may be less dominating in human skeletal muscle than in rat skeletal muscle and that more lipases than HSL are involved in TAG hydrolysis in the resting state. It has been shown that electrically induced muscle contractions increase the neutral lipase activity of soleus muscle of rats (Langfort et al. 2000). It is surprising from the point of view that exercise is accompanied by increases in circulating catecholamine concentrations, and there is evidence for activation of HSL by epinephrine. Thus, in incubated rat soleus muscle, HSL was activated by epinephrine (Langfort et al. 1999). The impact of skeletal muscle lipid metabolism on exercise and insulin resistance has been reviewed by Kiens (2006). The following assays can be used for investigation of the effects of compounds/drug candidates on the regulation and activity of muscle lipases.

K.6.2.17.3.1

Lipolysis in Isolated Muscle Strips

PROCEDURE

In studies of glycerol release, muscle strips are incubated for indicated periods of time (usually 90 min) in 2 ml of KRB buffer supplemented with 20 mg/ml BSA (pH 7.4), 1 mg/ml glucose, and 0.1 mg/ml ascorbic acid in a shaking water bath (37°C), with air as gas phase according to the protocol of Enoksson and coworkers (2005). After incubation, an aliquot of the medium is removed for analysis of glycerol using an ultrasensitive bioluminescent method (Hellmer et al. 1989). Strips are incubated in duplicate or triplicate in the absence (basal) or presence of various concentrations of isoproterenol, which is a non-selective β -adrenoceptor agonist. The muscle strip is removed from the medium and frozen in liquid nitrogen. It is then removed from the clamps and weighed. Lipolysis is expressed as millimole of glycerol per total incubation medium per time unit per milligram of muscle strip. In studies of FFA release, muscle strips are incubated and analyzed as described for glycerol release, except that 20 mg/ml of fatty acid-free BSA is used as the protein component in the incubation buffer, the number of strips in each type of incubation should range between five and ten, and FFA is analyzed by an ultrasensitive chemiluminescence method as described for adipocytes (Näslund et al. 1993; see K.6.1.10.5).

K.6.2.17.3.2**TAG Lipase Activity in Soleus Muscle****Incubation and Electrical Stimulation**

For electrical stimulation of the isolated soleus muscle after 1 h pre-incubation with compound/drug, the isolated soleus muscle with is transferred to fresh incubation medium and fixed in the vertical position and at resting length by small clips attached to the tendons according to the procedure introduced by Langfort and coworkers (2000). The upper tendon is connected to a force transducer. If subsequent electrical stimulation required, the muscles either remain resting or are stimulated electrically through electrodes in both ends to perform repeated ($1 \cdot s^{-1}$) maximal tetanic contractions (200-ms trains of 100 Hz, impulse duration 0.2 ms, 25 V) for 1, 5, 10 or 60 min while tension is recorded.

At the end of the incubation, muscles are freeze-clamped with aluminium tongs cooled in liquid nitrogen, and then trimmed of connective tissue and visible fat while kept in liquid nitrogen. In order to further ensure that findings reflected the biology of muscle cells, these are isolated before analysis from other tissue components by microdissection using a stereomicroscope after freeze-drying. Sometimes single fibres are isolated from fresh, non-frozen muscle by microdissection after collagenase digestion (one muscle being incubated for 3 h at 37°C in 5 ml of DMEM containing 2% (w/v) collagenase).

Preparation of Homogenates

Generally, muscles are homogenized (e. g. Polytron PT 3100, maximum speed) on ice in 10 vol. of 0.25 M sucrose, 1 mM DTT, 40 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 20 mM HEPES/KOH (pH 7.0), 0.31 μ M okadaic acid, 20 μ g/ml leupeptin, 10 μ g/ml antipain and 1 μ g/ml pepstatin. The crude homogenate is centrifuged ($15,800 \times g$, 1 min, 4°C). The supernatant is recovered and stored at -80°C until analysis within 1 week. In experiments evaluating the effect of phosphatase treatment on muscle homogenate, the homogenization buffer contained 20 mM Pipes (pH 7.0) instead of sodium pyrophosphate and glycerophosphate.

HSL Assay

HSL assays are based on measurement of release of [^3H]oleic acid from 1(3)-mono[^3H]oleoyl-2-oleylglycerol, a DAG analog not hydrolysable at position 2 (referred to as MOME activity), and from tri[^3H]olein (referred to as TOG activity). Upon phosphorylation by PKA, the TOG activity of adipose tissue

HSL increases markedly (Fredrikson et al. 1981, Cook et al. 1982), whereas the MOME activity does not change significantly (Cook et al. 1982, Cook et al. 1983, Greenberg et al. 2001, for a review see Yeaman 2004). Accordingly, MOME activity is a measure of the total enzyme concentration, whereas TOG activity is a measure of the activated form of HSL, and represents the assay of choice for monitoring changes in the activation state of HSL. The TOG and MOME substrates are emulsified with phospholipids by sonication, and BSA is used as fatty acid acceptor. Samples of 14 μ l (for TOG activity measurements) or 7 μ l (for MOME activity measurement) of muscle supernatant (protein concentration ~ 3 mg/ml) or pellet (resuspended to initial volume in homogenization buffer; protein concentration ~ 4.4 mg/ml) are incubated for 30 min at 37°C with 100 μ l of 5 mM TOG (1.25×10^6 cpm) or MOME (0.4×10^6 cpm) substrate and enzyme dilution buffer (to a total volume of 200 μ l). Hydrolysis is stopped by the addition of 3.25 ml of methanol/chloroform/heptane (10/9/7, by vol.), followed by 1.1 ml of 0.1 M potassium carbonate/0.1 M boric acid (pH 10.5). The mixture is vortexed vigorously for 10 s and centrifuged ($1,100 \times g$, 20 min). A 1-ml portion of the upper phase containing the released fatty acids is mixed with 10 ml of scintillation liquid. Radioactivity is determined in a scintillation counter.

K.6.2.17.3.3**Magnetic Resonance Spectroscopy Study of Intramyocellular Lipid Content**

Kuhlmann et al. (2003) performed a longitudinal *in vivo* ^1H -spectroscopic study of intramyocellular lipid content in Zucker diabetic fatty (ZDF) rats. Magnetic resonance spectroscopy (MRS) has been established as a dependable method for selective detection and quantification of intramyocellular lipid (IMCL) in humans. To validate the interrelation between insulin sensitivity and IMCL in an animal model of type 2 diabetes, volume-selective ^1H -MRS at 7 Tesla to non-invasively assess IMCL in the rat was established. In male obese ZDF rats and their lean littermates, IMCL levels were determined repeatedly over 4 months, and insulin sensitivity was measured by the euglycemic-hyperinsulinemic clamp method at 6–7 and at 22–24 weeks of age.

In vivo MRS studies were performed using a 7-T Biospec system (Bruker BioSpin, Ettlingen, Germany), a resonator for excitation and an actively decoupled surface coil for signal detection. Rats were anesthetized with 2–3 vol% isoflurane and 1:2

O₂:N₂O, and their temperature was kept at 37.5°C. The animals were fixed in a non-magnetic device allowing for accurate alignment of their hindleg on top of the surface coil. Voxels of ≈8 mm³ size were located in *M. soleus* and in *M. tibialis anterior*, avoiding vascular structures and gross adipose tissue deposits. Volume-localized ¹H-MR spectra [PRESS sequence, echo time (TE)=17 ms, repetition time (TR)=1 s, CHESS water suppression, 1024 averages] were obtained with Bruker's ParaVision acquisition software. The integral of the IMCL signal (1.3 ppm) was related to that of total creatine (tCr; 3.05 ppm; program MRUI 97.2, cf. <http://www.mrui.uab.es>). The IMCL/tCr ratio corresponded to the total muscle IMCL value. In all cases, a clear distinction between EMCL and IMCL was possible. As the IMCL/tCr ratio did not change on using a relaxation delay TR of 2 s instead of 1 s (*n*=4), the influence of relaxation time changes on observed IMCL values could be excluded. The tCr values for the *M. soleus* and *M. tibialis anterior* were determined in obese ZDF rats at 8 weeks and 15 months of age (*n*=8 each). In both muscles, tCr concentrations proved to be constant (soleus 89±1.1 and 81±1.7 μmol/g dry wt.; tibialis 136±2.2 and 132±1.0 μmol/g dry wt., respectively) and therefore to be a good reference for quantification of IMCL.

For assessing hepatic fat content, the rats were placed prone with their upper abdomen on top of the detecting surface coil. Volume-localized, respiration-triggered ¹H-MR spectra were obtained without water suppression (TE=28 ms, TR=1 s, 8 mm³). Fat content was expressed as the ratio of the fat-to-water signal (in percentage).

K.6.2.18

Determination of Other Metabolites in Muscle

Muscle non-lipidic metabolites are determined according to the procedure of Watt and coworkers (2004). For this, muscle samples are divided into aliquots under liquid nitrogen. One piece of muscle (~80 mg wet muscle) is freeze-dried, dissected free of non-muscle contaminants under magnification, and divided into four aliquots. Muscle for glycogen analysis (3 mg) is extracted in 2 M HCl and neutralized with 0.67 M NaOH, and glycogen content is determined as described above and adapted from Passoneau and Landerdale (1974). A second aliquot of muscle (2 mg) is extracted according to Harris and coworkers (1974), and ATP, phosphocreatine, creatine, and lactate are determined by enzymatic fluorometric techniques according to well-established procedures (Passoneau and

Lowry 1993). Intramyocellular TAG content is determined by extraction of the TAG from ~6 mg of tissue in chloroform/methanol (2/1), saponification of the TAG in an ethanol-KOH solution at 60°C, and fluorometric determination of glycerol (Froberg and Mossfeldt 1971, 1975).

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K.6.3

Assays for Insulin and Insulin-Like Signal Transduction Based on Adipocytes, Hepatocytes and Myocytes

GENERAL CONSIDERATION

After having established insulin-like activity of compounds/drug candidates in primary or cultured adipose, muscle, and liver cells or tissues with one or several of the metabolic assays described above (see K.6.1 and K.6.2), it is often useful to elucidate the molecular mode of action of these compounds/drug candidates for further characterization and optimization, in particular regarding selectivity and potency. For this, detailed knowledge in the molecular mechanisms of the insulin signal transduction cascade as well as of cross-talking insulin-like signaling pathways as well as the availability of appropriate reliable and robust cell-free and cell-based assays reflecting these events are required. The following view results from the current experimental findings but due to limitations in space and rapid progress still made in this area has to be considered as simplified and temporary, only.

Upon binding of insulin to the IR (insulin receptor) in the major insulin-responsive target cells (adipocytes, myocytes, hepatocytes), the insulin signal transduction machinery, a complex network of protein-protein interactions and protein (serine/threonine/tyrosine) phosphorylation and dephosphorylation cascades between a multitude of cellular signaling components (e. g. enzymes, adaptor proteins, structural proteins), is activated and transmits the insulin signal to a variety of metabolic (predominantly) and mitogenic (to a minor degree) effector systems (e. g. glucose transport, GLUT4 translocation, glycogen synthesis, gene expression, cell differentiation, DNA synthesis) (Biddinger and Kahn 2006, Myers and White 1995, Saltiel 1996, Yenush and White 1997, White 1998, Watson and Pessin 2006). For compounds/drug candidates with insulin-like/sensitizing activity, it is important to differentiate between metabolic and mitogenic signaling in cells of both high (e. g. adipocytes) and low insulin responsiveness (e. g. fibroblasts).

Two major pathways within the insulin signaling cascade have been dissected and are thought to mediate the different biological functions of the hormone: (I) Activation of phosphatidylinositol-3'-kinase (PI3-K) plays a pivotal role for regulation of glucose transport and cellular trafficking as well as glycogen synthesis and lipolysis by insulin. (II) Formation of

the Shc-Grb2 complex leads to activation of the Ras-pathway which has been linked to insulin regulation of both cell growth and gene expression, although this has been questioned recently. Many of the proteins involved in these two cascades have been identified at the molecular level (White 1997, Holman and Kasuga 1997, White 2002). The most upstream located one, the insulin receptor (IR) is a transmembrane tyrosine kinase which when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates a number of intracellular substrates, among them one or more 50- to 60-kDa proteins, including Shc, a 15-kDa fatty acid binding protein and several so-called insulin receptor substrate proteins, IRS-1/2/3/4, and Gab, which act as adaptor proteins lacking any enzymatic activity. Following tyrosine phosphorylation, the phosphotyrosine residues of the IRS polypeptides act as docking sites for several Src homology 2 (SH2) domain-containing adaptor molecules and enzymes, including PI3-K, Grb2, SHP2, Nck, and Fyn. The interaction between the IRS proteins and PI3-K occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit.

PI3-K is essential and may even be sufficient for many of the insulin-regulated metabolic processes, including stimulation of glucose transport and glycogen synthesis and inhibition of lipolysis (Yeh et al. 1997, Alessi and Downes 1998, Shepherd et al. 1998, Shepherd 2005, Cheatham et al. 1994, Herbst et al. 1995, Yeh et al. 1997). PI3-K activation results in the production of phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (3,4)-bisphosphate which leads to binding and concomitant activation of the membrane-associated serine/threonine protein kinases, PDK1/2 (the nature of PDK2 remaining unclear so far). The binding of the pleckstrin-homology domain of PKB to the PI3-K-generated phosphoinositides both recruits PKB to the plasma membrane and *via* phosphorylation by PDK1/2 stimulates its kinase activity. Activation of Akt requires that it undergoes phosphorylation at two sites (Welsh et al. 2005). PDK1 phosphorylates Akt at Thr308, a residue located in its kinase-domain activation loop. In addition, Ser473 in the C-terminal hydrophobic motif of Akt has long been known to undergo phosphorylation but the identity of the kinase responsible has been controversial. Recently, however, an enzyme complex consisting of mTOR (mammalian target of rapamycin) and RICTOR (rapamycin insensitive companion of mTOR) has been shown to phosphorylate Akt at Ser473 in response to insulin (Hresko and Mueckler 2006, Sarabassov et al. 2005).

Thus the current knowledge favors the view that most metabolic insulin signals emerge from insulin-dependent tyrosine phosphorylation of the IRS proteins (and additional adaptor proteins located in lipid rafts, see K.6.3.6), whereas the mitogenic insulin action is apparently coupled to tyrosine phosphorylation of the Shc proteins. The latter serve as docking sites for the Grb-SOS complex which possesses GDP-GTP exchange activity for the small G-protein Ras and is activated by binding to Shc. In turn GTP-loaded Ras interacts with and activates the Raf serine kinase which phosphorylates the dual specificity kinase, mitogen/extracellular signal-activated kinase MEK (=MAPKK). Activated MEK phosphorylates the mitogen-activated protein kinases MAPK, ERK1 (p44) and ERK2 (p42), which in turn phosphorylate and activate a number of transcription factors (e. g. c-jun, c-fos) and structural proteins ultimately leading to increased gene and protein expression as well as DNA synthesis in insulin-like fashion (Gustafson et al. 1998).

In addition to the identification of the signal transduction pathways directly leading from the IR to downstream targets, several cross talks have been delineated between signal transmission by insulin and other hormones/growth factors (e. g. EGF, angiotensin) or diverse exogenous stimuli (e. g. H₂O₂, phosphoinositolglycans, see K.6.3.6.4) which either mimic to a certain degree (insulin-like activity) or modulate in a positive or negative fashion (insulin-sensitizing or desensitizing activity) metabolic and/or mitogenic insulin action in various cellular systems. Since none of these ligands activates the IR kinase directly, their signalling pathways may converge with that of insulin at a more distal signalling step (Argetsinger et al. 1995, Huppertz et al. 1996, Kowalski-Chauvel et al. 1996, Velloso et al. 1996, Verdier et al. 1997, Baron et al. 1998). Soluble phosphoinositolglycan molecules (PIG) which have been shown to exert partial insulin-like effects in diverse cellular and subcellular systems (Müller et al. 1997, Kessler et al. 1998, Frick et al. 1998, Leon and Varela-Nieto 2004, Müller et al. 2005) can also be classified into the latter category. Interestingly, the second-generation sulfonureas, glibenclamide and, in particular, glimepiride have been demonstrated to stimulate glucose transport and non-oxidative metabolism in adipose and muscle cells *in vitro* by causing IR-independent tyrosine phosphorylation of IRS-1/2 and stimulating the downstream located insulin signaling events (Müller et al. 1994, Müller and Geisen 1996, Müller 2005). This insulin-like signaling in cells of peripheral tissues

may explain the insulin-independent blood glucose-lowering activity of glimepiride/glibenclamide as has been reported in a number of animal studies (Geisen 1988, Müller et al. 1995, Müller 2000, 2005), which supplements the potent blood glucose decrease *via* insulin release provoked by these widely used anti-diabetics.

K.6.3.1

Insulin Receptor (IR) Activation

GENERAL CONSIDERATIONS

Insulin exerts its biological effects through a plasma membrane receptor that possesses a tyrosine kinase activity. Binding of insulin to the α -subunits of the IR induces autophosphorylation *in trans* on tyrosine residues of its β -subunits and thereby stimulates the intrinsic tyrosine kinase activity toward intracellular substrates (such as IRS proteins and Shc) that play crucial roles in the transmission of the signal (see K.6.3). Alterations in tyrosine phosphorylation of the IR have been described in insulin resistant states, such as diabetes and obesity (Combettes-Souverain and Issad 1998). The discovery of pharmacological agents that specifically activate the tyrosine kinase activity of the IR in cell-free and cell-based assay systems has recently been reported (Zhang et al. 1999) and apparently formed the starting point for a number of drug discovery efforts for future insulin-like or sensitizing drugs for the treatment of insulin-resistant patients.

The first critical node in the insulin-signaling network is, by definition, IR and the associated IRS. The IR is a tetrameric protein that consists of two extracellular α -subunits and two intracellular β -subunits. It belongs to a subfamily of receptor tyrosine kinases (RTKs) which also includes the insulin-like growth factor-1 receptor (IGF1R) and an orphan receptor, known as the IR-related receptor (IRR). Each of these receptors is the product of a separate gene, in which the two subunits are derived from a single-chain precursor or proreceptor that is processed by a furin-like enzyme to give a single α - β dimers linked with disulfide bonds to form the tetrameric $\alpha_2\beta_2$ holoreceptor. This configuration allows for the creation of a hybrid IR-IGF1R complex, which serves as an additional receptor isoform modulated by insulin-like/sensitizing stimuli in differential fashion. The activity of the IR is tightly regulated, as unchecked activation or inactivity would lead to profound metabolic (and presumably proliferative) consequences. There are two splice isoforms of the IR, and each has a different affinity for insulin and IGF-1 (DeMeyts 1976).

Functionally, the IR behaves like a classical allosteric enzyme in which the α -subunit inhibits the tyrosine-kinase activity that is intrinsic to the β -subunit. Insulin binding to the α -subunit, or removal of the α -subunit by proteolysis or genetic deletion, leads to partial derepression, i. e. activation of the kinase activity in the β -subunit by stabilizing the open/relaxed rather than the closed/tense conformation of the activation loops of the two receptor halves. Following this initial activation, transphosphorylation of the β -subunits at tyrosines 1158, 1162, and 1163, leads to an additional stabilization of this conformational change even in the absence of ongoing insulin binding (Ellis et al. 1986.). The determination of the crystal structure of the tyrosine kinase domain of the human IR has provided a better understanding of the molecular mechanism involved in the stimulation of its kinase activity. In the unphosphorylated state, Tyr-1162 is located in the active site of the enzyme and plays an autoinhibitory role by competing with the binding of protein substrates. This tyrosine remains in the unphosphorylated form in the basal state, because other residues in the activation loop also impair ATP binding. The crystallization of the tris-phosphorylated form of the kinase domain has shown that autophosphorylation of these three tyrosines results in a dramatic change in the conformation of the activation loop (Hubbard 1997). This conformation change permits unrestricted access to the binding sites for ATP and protein substrates. It has been postulated that conformational changes induced by ligand binding move the kinase domain of the two β -subunits of the IR nearer to each other, thereby allowing *trans*-phosphorylation of tyrosine 1162 and adjacent tyrosines in the activation loop.

It is important to note that unlike other RTK that bind directly to the cytoplasmic tails of downstream effectors, the IR and IGF-1R engage specific proteins that are known as the IRS proteins and mediate the binding of intracellular effectors (see above). In addition, the IR and the IRS proteins share common mechanisms of regulation: They are negatively regulated by dephosphorylation by protein tyrosine phosphatases (PTPs), phosphorylation by serine/threonine kinases which interfere with IR/IRS function by sterically blocking interaction of IR and IRS or modifying IR kinase activity or downstream signaling of IRS, binding of inhibitory adaptor and signaling proteins (e. g. suppressor of cytokine signalling SOCS1/3, plasma membrane glycoprotein PC-1) and ligand-induced down-regulation of IRS at the translational and transcriptional level. Furthermore, the IR is also down-regulated at the protein level by ligand-induced inter-

nalization and degradation. Some of these regulatory processes may be dysregulated during the pathogenesis of insulin-resistant, hyperinsulinemic states, including obesity, type II diabetes and metabolic syndrome. The following methods may be helpful for the identification and characterization of insulin-like and sensitizing compounds/drug candidates as well as of insulin analogs with favorable kinetic (short- or long-lasting) action profiles using cell-free and cell-based assay systems.

K.6.3.1.1

Insulin Binding

PURPOSE AND RATIONALE

IR binding studies have been performed with various animal tissues and primary and cultured cells of rodent and human origin. Human adipocytes can be used to study simultaneously IR binding and metabolic effects of insulin (Hjöllund 1991). The binding tests are of value to characterize newly synthesized insulin analogs (Schwartz et al. 1987, Ribel et al. 1990, Vølund et al. 1991, Robertson et al. 1992).

K.6.3.1.1.1

Preparation of ^{125}I -Insulin

Most investigators use the "chloramine-T procedure" to iodinate insulin. The reaction is carried out in a 20 ml glass vial in an ice-bath with continuous magnetic stirring. To 2.5 ml 0.05 M phosphate buffer (pH 7.5), 2.0 mCi Na^{125}I , and 15 μl of a 1 mg/ml insulin solution are added. Then, 0.5 ml of a chloramine T (50 mg/ml) solution is added dropwise over the course of 1 min. After 10 min, 0.7 ml of a freshly prepared sodium metabisulfite solution (50 mg/ml in 0.05 M phosphate buffer, pH 7.5) is added. One ml of this reaction mixture is transferred to 10 ml 2% BSA for determination of specific activity. In order to absorb unreacted ^{125}I and damaged products, 2.0 g 20–50 mesh AG 1X-8 resin (BioRad) are added (equilibrated in 1 ml 0.05 M phosphate buffer, pH 7.5, containing 0.1 mg/ml thiomersol and 20 mg/ml crystalline BSA). The reaction mixture is stirred for 10 min, decanted from the resin and diluted to a concentration of less than 25 $\mu\text{Ci/ml}$ in a solution of 0.8 M glycine, 0.2 M NaCl, 0.05 M phosphate (pH 7.5), and 2.5 mg/ml crystalline BSA. The final solution is stored in multiple aliquots at -70°C .

K.6.3.1.1.2

Binding to Purified Insulin Receptor

IR are partially purified by affinity chromatography on WGA-Sepharose according to the procedure intro-

duced by Ellis and coworkers (1986). Confluent flasks of cells are solubilized with 50 mM HEPES/KOH (pH 7.6), 1 mM PMSF, 1 mg/ml bacitracin. The lysate is then centrifuged ($100,000\times g$, 1 h, 4°C) and loaded on a 2-ml WGA-Sepharose column for 2 h at 4°C . After washing of the column with buffer C (50 mM HEPES/KOH, pH 7.6, 150 mM NaCl, 0.1% TX-100), bound proteins are eluted with 0.3 M N-acetyl-D-glucosamine in buffer C. The IR is further affinity-purified batch-wise by adsorption to monoclonal anti-hIR antibodies which are subsequently bound to anti-mouse IgG coupled to Sepharose beads. After several washing cycles with buffer C ($10,000\times g$, 2 min, 4°C), portions of the hIR-mAb-anti-IgG-beads are suspended in 50 μl of 20 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 20 mg/ml BSA, 0.05% TX-100 and then incubated with ^{125}I -labeled insulin (300 mCi/mg, 0.2 μCi , 5 pM) in the presence of increasing concentrations of unlabeled insulin for 1 h at 4°C . Subsequently, the hIR-mAb-anti-IgG-beads are collected by centrifugation ($10,000\times g$, 2 min, 4°C), rapidly washed with 500 μl of buffer C containing 20 mg/ml BSA and finally counted in a gamma-counter.

K.6.3.1.1.3

Binding to Cultured Cells

Chinese hamster ovary (CHO) cells expressing the wild-type human IR (CHO-hIR) are maintained in Ham's F12 medium supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$) (F12 medium). Cells ($\sim 10^6$ cells/100-mm plate) are cotransfected with plasmid DNA (10 μg of expression plasmid *plus* 2 μg of pSV2NEO) in the form of a calcium phosphate precipitate with the addition of a glycerol shock (20% glycerol in F12 medium) after 4 h. After 36 h, the cells are split 1:20 and allowed to grow for 24 h prior to addition of G418 (400 $\mu\text{g/ml}$). 10–14 d later, colonies from 5 plates (~ 250 –500 colonies) are harvested with trypsin/EDTA (0.05% trypsin, 0.02% EDTA in Ca^{2+} -free and Mg^{2+} -free Hank's basic salt solution and are replated in 24-well culture plates in F12 medium. 2 d later, semi-confluent cells ($\sim 4\times 10^4$ cells/well) are washed twice with buffer B (100 mM HEPES/KOH, pH 7.8, 120 mM NaCl, 1.2 mM MgSO_4 , 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose, 1% BSA) and are then incubated with 16 h at 4°C with 0.5 ml of buffer B containing ^{125}I -labeled insulin (30,000 cpm, 120 Ci/g) *plus* increasing concentrations of unlabeled insulin. Cells are washed twice, solubilized with 0.5 ml of 0.03% SDS and counted in a γ -counter.

K.6.3.1.1.4**Binding to Human Primary Adipocytes****PROCEDURE**

Subcutaneous adipose tissue (~4–5 g) is obtained from the abdomen of patients undergoing gastrointestinal surgery. Patients suffering from any endocrine or metabolic disorder or taking drugs known to affect metabolism should be excluded. Other exclusion criteria are impaired glucose tolerance measured by determination of fasting blood glucose and the 2 h value after a 75-g oral glucose load. The adipose tissue is finely chopped and incubated for 90 min at 37°C in 25 mM HEPES/KOH (pH 7.4), containing HSA (25 g/l) and collagenase (0.5 g/l). The isolated adipocytes are subsequently washed five times in HEPES/KOH (see above) containing 50 g/l HSA.

IR binding studies with isolated human adipocytes are performed in a 300 µl cell suspension containing about 1×10^5 cells/ml in 10 mM HEPES (pH 7.4), 50 g/l HSA at 37°C. The iodine labeled ligand ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$ -monoiodinated insulin, specific activity about 350 mCi/mg) in a final concentration of 20 pM is incubated with increasing amounts of unlabeled human insulin and the insulin analog/compound/drug candidate to be tested. The reaction is stopped by adding 10 ml of chilled 0.150 M NaCl and subsequent centrifugation through silicone oil (Pedersen et al. 1981 and 1982, Zeuzem et al. 1984). Non-specific binding is measured by incubating tracer in the presence of a large excess of unlabeled insulin.

For kinetic analysis of binding, the association is studied by incubation of the ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$ -monoiodinated insulin for various times (1 to 240 min) and the reaction is terminated as described above. At each time point, the non-specific binding is measured and subsequently subtracted from the corresponding data for total binding. Dissociation rates are determined by first incubating isolated human adipocytes at 37°C with either $[^{125}\text{I}]\text{Tyr}^{\text{A14}}$ -insulin or the test compound labeled in the same position for 90 min to achieve steady-state binding conditions. Each incubation mixture is then centrifuged for 60 s. The adipocytes are rapidly washed twice by diluting with buffer to the original volume at 4°C and the centrifugations and aspirations are repeated. After the third aspiration, the cells are diluted to the original volume with buffer alone or native insulin or the insulin analog or compound to be tested at a final concentration of 0.2 µM at 22°C. At this hormone concentration a maximal effect of ^{125}I -insulin dissociation is reported (Podlecki et al. 1984, DeMeyts et al. 1976). The reaction is

stopped at various times between 10 and 180 min and cell-associated radioactivity is determined.

K.6.3.1.2**IR Conformational Change Using BRET and FRET****PURPOSE AND RATIONALE**

BRET is a natural phenomenon in which resonance energy transfer occurs between luminescent donor and fluorescent acceptor proteins (Xu et al. 1999, Angers et al. 2000). Resonance energy transfer occurs when part of the energy of an excited donor is transferred to an acceptor fluorophore, which re-emits light at another wavelength. Resonance energy transfer only takes place if the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule overlap sufficiently and if the donor and the acceptor are in proper distance (10–100 Å) and relative orientation (Tsien et al. 1993, Wu and Brand 1994). In the BRET methodology, the first protein partner is fused to *Renilla* luciferase (Rluc), whereas the second protein partner is fused to a fluorescent protein (e. g. yellow fluorescent protein YFP). If the two partners do not interact, only one signal, emitted by the luciferase, can be detected after addition of its substrate coelenterazine. If the two partners interact, resonance energy transfer occurs between Rluc and the YFP, and an additional signal, emitted by the YFP, can be detected. The strict dependence of BRET on the molecular proximity between energy donors and acceptors makes it a system of choice to study changes in the interaction between two proteins, which usually (in the unstimulated state of the target cell) exist as separate entities or are already assembled in a preformed complex susceptible to changes in their relative neighborhood or orientation in response to appropriate stimuli.

Binding of insulin to the IR induces a conformational change that brings the two β -subunits of the IR into close proximity, allowing trans-phosphorylation of one IR β -subunit by the other subunit (Hubbard 1997, Combettes-Souverain and Isaad 1998). This autophosphorylation stimulates the tyrosine kinase activity of the IR towards intracellular substrates, such as IRS-1 and initiated downstream signaling to the terminal metabolic insulin effector systems, such as GLUT4 translocation, which are impaired in insulin-resistant and diabetic states. Therefore, methods that allow monitoring of the activity of the IR constitute important tools for the search for insulin-like compounds and putative anti-diabetic drugs.

Biazzo-Ashnault and coworkers (2001) developed a FRET-based assay for monitoring IR kinase activ-

ity. In this assay, the IR has to be immobilized on a microtiter plate with anti-IR antibody. A kinase reaction is then performed using a phosphorylation cocktail containing a biotinylated substrate. After incubation with a fluorescent donor, a streptavidin-labeled fluorescent acceptor that to the biotinylated substrate is added. Energy transfer only occurs if the fluorescent donor coupled to anti-phosphotyrosine antibody is in close proximity to the streptavidin-labeled fluorescent acceptor, i. e. if the peptide substrate is phosphorylated on tyrosine residues. Obviously, this is still a heterogeneous assay involving time-consuming incubations and washing steps.

By contrast, Boute and coworkers (2001) developed a completely homogeneous test, based on the BRET methodology, to follow IR kinase activity. A chimeric human IR, in which one IR β -subunit is fused to *Renilla* luciferase (Rluc) and the other IR β -subunit is fused to yellow fluorescent protein (YFP) is produced in HEK cells and partially purified by wheat germ lectin chromatography. Insulin-induced conformational change could be detected as an increase in energy transfer (BRET signal) between Rluc and YFP. This BRET signal corresponds to the ligand-induced conformational changes of the IR before any phosphorylation event, and faithfully reflects the activation state of the IR (Boute et al. 2001).

PROCEDURE

Cell-Based Assay

HEK-293 cells maintained in DMEM supplemented with 4.5 g/l glucose and 10% FBS (Invitrogen) are seeded at a density of 1.2×10^6 cells per 100-mm dish. Transient transfection is performed 1 day later using FuGene 6 (Roche Diagnostics) with either 0.3 μ g of IR-Rluc cDNA and 0.3 μ g of empty vector or with 0.3 μ g of IR-Rluc and 0.3 μ g of IR-YFP cDNAs per 100-mm dish. BRET measurements on intact cells are performed essentially as described by Angers and coworkers (2000). 2 days after transfection, HEK-293 cells are detached with Trypsin-EDTA and resuspended in PBS. Approximately 60,000 cells per well are distributed in a 96-well microplate. Cells are incubated for 2 to 10 min in absence or presence of 100 nM insulin. Coelenterazine is added at a final concentration of 5 μ M, and light-emission acquisition is started immediately. For use of adherent cells, cells are transfected exactly as described above, but 1 day after transfection, cells are transferred into 96-well microplates (white CulturPlate-96, Packard) at a density of 30,000 cells per well.

In Vitro Assay on Partially Purified Fusion IR

BRET measurements are performed using partially purified fusion IR. Two days after transfection, cells are extracted in buffer containing 1% Triton X-100, 20 mM MOPS, 2.5 mM-benzamidine, 1 mM-EDTA, 1 mM-4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 1 μ g/ml each of aprotinin, pepstatin, antipain, and leupeptin. Fusion receptors are partially purified by chromatography on wheat-germ lectin Sepharose by Tavare and Denton (1988). Partially purified fusion IR are aliquoted and stored at -80°C for subsequent use. *In vitro* measurement of BRET signal is performed using 4.5 μ l of wheat-germ lectin (WGL) eluate (approximately 2 μ g of proteins) preincubated in 96-well microplates for 45 min at 20°C in a total volume of 60 μ l containing 30 mM MOPS, 1 mM Na_3VO_4 , and different concentrations of ligands. Coelenterazine (7 μ l, 2.6 μ M final concentration, Molecular Probes, USA) is added to the preparation and light emission acquisition at 485 nm (filter window 20 nm) and 530 nm (filter window 25 nm) is started immediately using a microplate analyser (e. g. Fusion, Packard).

EVALUATION

The BRET ratio has been defined previously (Angers et al. 2000) as $[(\text{emission at } 530 \text{ nm}) - (\text{emission at } 485 \text{ nm}) \times \text{Cf}] / (\text{emission at } 485 \text{ nm})$, where Cf corresponds to $(\text{emission at } 530 \text{ nm}) / (\text{emission at } 485 \text{ nm})$ for the Rluc fusion protein expressed alone in the same experimental conditions (i. e., IR-Rluc transfected alone).

The BRET signal detected in this assay closely reflects the activation state of the IR independently of any phosphorylation reaction. It is a completely homogeneous procedure, requiring no washing step. Thus, the procedure allows for very rapid determination of the activation state of the IR and can be easily used in throughput screening tests for the search of novel compounds/drug candidates with insulin-like activity. Indeed, partially purified fusion IR can be prepared on a large scale by WGL-chromatography and stored at -80°C for subsequent use. This preparation can be distributed in an automated way in 96-well microplates and the effect of molecules on IR activity can be measured within a few minutes using the BRET method. Therefore, the same preparation can be used for multiple screening rounds. Similar procedures should be applicable to any receptor in which ligand binding induces dimerization or conformational changes de-

tectable by BRET. Thus, this assay could be a valuable tool for the search of compounds/drug candidates with anti-diabetic properties.

For screening purposes it turned out that the *in vitro* rather than the cell-based assay has to be used (Boute et al. 2001). The chimeric IR are correctly expressed and functional in HEK293 cells, but an unexpected high basal BRET signal was observed. Obviously, the choice between an *in vivo* and *in vitro* assay has to be done on a case-by-case basis. However, it must be kept in mind that in an intact cell assay, cells have to be distributed and cultured in microtiter plates for each screening round, which is highly time-consuming.

Comparison with FRET Technology

Before the development of BRET in 1999, fluorescence resonance energy transfer (FRET) has been used widely to study protein-protein interactions or to monitor conformational changes within a given protein. In the FRET methodology, luciferase is replaced by a fluorophore that is excited using monochromatic light at the appropriate wavelength (Cubitt et al. 1995, Hovius et al. 2000, Zacharias et al. 2000). A major advantage of FRET is to permit, under microscopic observation, visualization in a single living cell of protein-protein interactions at the subcellular level (Wouters et al. 2001). This is difficult to perform with the present version of the BRET methodology because of the low intensity of light emission by luciferase.

However, FRET presents several disadvantages that make BRET a technology of choice in many cases. In FRET, the donor fluorophore must be excited using monochromatic light. Because of the overlapping absorption and emission spectra of the existing mutants of GFP, direct excitation of the acceptor fluorophore by the light used to excite the donor often complicates the interpretation of the results. This obviously does not occur in BRET because excitation of the donor partner does not require exogenous illumination. Therefore, the quantification of resonance energy transfer is much easier in BRET than in FRET (Tsien 1998). Another related advantage of BRET is that the relative levels of expression of donor and acceptor partners can be quantified independently by measuring the luminescence of the donor and the fluorescence of the acceptor (Xu et al. 2002). Moreover, in FRET, excitation of the donor by illumination of the sample can induce photobleaching of the donor fluorophore, which results in loss of signal with time (Tsien et al. 1993, Cubitt et al. 1995). In addition, in FRET, illumination of the sample often induces autofluorescence that is emitted by endogenous cell components. The resulting back-

ground can easily be dealt with when FRET is assessed in a single cell with imaging techniques, by using an unlabeled region of the cell as an internal reference for the autofluorescence background (Tsien 1998). However, this background might become a serious problem when interactions are measured on populations of cells or molecules using non-imaging methods, such as fluorometry in microtiter plates (as would be desirable for throughput screening assays). Recent studies indicate that a tenfold improvement in sensitivity could be attained by using BRET rather than FRET, in an otherwise identical assay (Arai et al. 2000, 2001). Finally, it must be taken into account that the instrumentation requirements for BRET are simpler than those for FRET, which needs an excitation light source. A comparative evaluation of BRET vs. FRET technologies with emphasis in their putative application in high-throughput screening has been provided by Boute and coworkers (2002). A chimeric human insulin receptor, in which one-receptor β -subunit was fused to *Renilla* luciferase and the other receptor β -subunit was fused to yellow fluorescent protein, was produced in HEK cells and partially purified by wheat germ lectin chromatography. Insulin-induced conformation change could be detected as an increase in energy transfer (BRET signal) between *Renilla* luciferase and the yellow fluorescent protein.

K.6.3.1.3

IR Tyrosine Phosphorylation

K.6.3.1.3.1

Phosphorylation with Purified IR

PROCEDURE

Preparation of Purified IR

IR is extracted and purified from human placenta as described by Yamaguchi and coworkers (1983) with modifications from Ozawa and coworkers (1998). After preparation of placental membranes from one piece of fresh normal human placenta by differential centrifugation, a suspension containing the membrane proteins is mixed with the same volume of a 50 mM Tris/HCl buffer (pH 7.4) containing 2% Triton X-100 and protein inhibitors (2 mM BAEE, 1 mg/ml pepstatin, 1 mg/ml aprotinin, 1 mg/ml leupeptin and 0.1 mM PMSF) for 45 min at 4°C with stirring. A clear supernatant is obtained by centrifugation (100,000 × g, 90 min, 4°C) and is transferred to 40 ml of a WGA-Sepharose affinity column. The fractions including the protein are pooled and applied to 6.3 ml of an insulin-Sepharose column. After thorough washing of the column with 50 mM Tris-HCl (pH 7.4) containing 1.0 M

NaCl, 0.1% Triton X-100 and 0.1 mM PMSF, the IR is eluted with 50 mM acetate buffer (pH 5.0) containing 1.0M NaCl and 0.1% Triton X-100. The active fractions assessed by ^{125}I -insulin binding activity are collected and concentrated by pressured dialysis using a Diaflo ultrafiltration membrane PM-30 (Amicon Inc.). The purified IR is stored at -80°C . The purity of the IR is assessed by SDS-PAGE and silver staining. The purified IR results in two bands of 135 and 90 kDa, which correspond to α - and β -subunits of the IR, respectively. The stability of the purified IR is determined by the kinase activity of IR at various time intervals after elution of the insulin-Sepharose column. There is no apparent change for the kinase activity up to 1 month, which is evaluated from the magnitude of phosphorylated artificial substrate, poly(Glu 80 Tyr 20), incubated with insulin and its receptor. The IR prepared by the present method is thus of sufficient stability. Kinase activities of purified IR are typically found to change from one extraction to another. In order to control this kinase activity so that it would be the same throughout the experiment, the concentration of the purified IR has to be adjusted by dilution before the IR is applied for measurements in the present method.

Radioactive Assay

The direct effect of insulin/compounds/drug candidates on the purified IR is assessed by using random copolymers of (Glu 80 Tyr 20) $_n$ serving as a substrate for the IR kinase according to Braun and coworkers (1984). The purified IR is pre-incubated with 0.48 μM insulin at 22°C for 1 h in 630 μl of a solution (solution A) containing 25 μM ATP and 5.0 mM MnCl_2 . The random copolymer of (Glu 80 Tyr 20) $_n$ is dissolved in a buffer solution (solution B) containing 0.1% Triton X-100, 5.0 mM MnCl_2 , 10 mM MgCl_2 , 50 μCi [γ - ^{32}P]ATP, and 50 mM HEPES/KOH (pH 7.4) to its final concentration of 0.5 mg/ml. A 30- μl portion of solution B is taken into a microtube. Phosphorylation of (Glu 80 Tyr 20) $_n$ is initiated by adding 15 μl of the pre-incubated IR solution into the microtube, and the solution is incubated for 2 to 120 min. After each incubation time, the sample is applied to filter paper (Whatman 3MM). The filter paper is washed 6 times with 10% TCA and then with acetone and finally air-dried. Radioactivity is counted with a liquid scintillation counter. The magnitude of phosphorylated random copolymers incubated with maximally effective concentrations of insulin is about 4- to 6- times higher than that without insulin at each time, demonstrating the activity of the IR preparation by the present

purification method. The insulin-like activity of compounds/drug candidates is calculated as percentage of the maximal insulin effect.

K.6.3.1.3.2

Phosphorylation with Intact Cells

K.6.3.1.3.2.1

Detection by Immunoblotting

IR is immunoprecipitated from cell lysates (9×10^6 cells) by incubating with rabbit anti-IR antibody (3 μg) in buffer A (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium pyrophosphate, 100 μM pervanadate, complete protease inhibitor mix tablets) overnight at 4°C with rotating. Prewashed protein G-sepharose beads (50 μl of packed beads) are added and the solution is mixed in a rotator for 2 h at 4°C . The beads are washed three times in buffer A and protein is eluted using SDS sample buffer (65 mM Tris/HCl, pH 6.8, 1.3% SDS, 13% glycerol, and bromophenol blue) with heating at 95°C for 5 min. SDS-PAGE is carried out by the method of Laemmli on 10% polyacrylamide gels using a mini-apparatus (e. g. Novex) in 25 mM Tris base, pH 8.3, 192 mM glycine, 0.1% SDS at 140 V constant voltage. Electrophoretic transfer from SDS-polyacrylamide gels to polyvinylidene fluoride (PVDF) (antiphosphotyrosine probing) or nitrocellulose (anti-IR probing) is carried out in a mini-blot apparatus (e. g. Novex). The blotting conditions are 40 V for 2 h in 12 mM Tris base, pH 8.3, 96 mM glycine, 20% methanol. Membranes are blocked in 1% polyvinylpyrrolidone (PVP-40, PVDF membranes) or 5% non-fat milk (nitrocellulose) and washed in 25 mM Tris (pH 7.2), 150 mM NaCl, 0.1% Tween 20. Phosphotyrosine-containing proteins are detected using antiphosphotyrosine (4G10) antibody-HRP-conjugated diluted 1:2,000 in 1% PVP-40. The β -subunit of the IR is detected using rabbit anti-IR diluted 1:1,000 in 5% milk followed by 1:2,000 dilution of anti-rabbit-HRP. Reactive proteins are visualized using chemiluminescent detection and a LumiImager.

K.6.3.1.3.2.2

Detection by ELISA

PURPOSE AND RATIONALE

A new assay method has been introduced by Ozawa and coworkers (1998) for the identification and evaluation of compounds/drug candidates with insulin-like activity and interfering with the insulin signaling pathway. It is based on the on/off switching mechanism of the IR-mediated insulin signaling. The Y939 sub-

strate peptide, a synthetic peptide of 12 amino acid residues, consists of a tyrosine-phosphorylated domain of IRS-1 and of a binding domain of IRS-1 to PI-3K. The Y939 peptide is immobilized on an avidin-coated 96-well microtiter plate. Upon binding of insulin to the IR prepared in total or immunoprecipitated from lysates of treated cells, insulin stimulates its receptor kinase activity in a sample solution and phosphorylates the tyrosine residue of the Y939 peptide immobilized on the well surface. The sample solution is washed out, and a selective binding protein for the phosphorylated tyrosine, monoclonal anti-phosphotyrosine antibody labeled with horseradish peroxidase, is added and forms a phosphorylated peptide-antibody complex. The amount of complex thus formed is detected by enzymatic amplification with horseradish peroxidase that can produce a green-color product with an absorbance at 727 nm capable of being detected by colorimetry. The amount of phosphorylated Y939 peptide thus measured is expected to be a selective and sensitive measure of the extent of the insulin signaling induced by compounds/drug candidates.

PROCEDURE

Preparation of Biotin-Y939 Substrate Peptide

The Y939 substrate peptide is biotinylated by reaction of a maleimide group of biotin-PEAC₅-maleimide with a thiol group of cysteine introduced at the carboxy-terminal end of the Y939 peptide. 1.0 mg of Y939 peptide (7.2×10^{-7} mol) is dissolved in 160 μ l of PBS buffer (150 mM NaCl, 3.0 mM KCl, 10 mM phosphate buffer, pH 7.2), and 2.5 mg of biotin-PEAC₅-maleimide (3.6×10^{-6} mol) dissolved in 40 μ l of DMSO is added dropwise to the Y939 peptide solution with gentle stirring. The reaction mixture is left for 12 h at 25°C. The reactants are purified by reverse phase HPLC performed on a 801-SC system (Japan Spectroscopic Co., Japan), equipped with a Kaseisorb LC ODS-300-5 column and a UV detector, using a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid at a constant flow rate of 1.0 ml/min over 60 min. The eluents are monitored by UV absorbance at 220 nm. 10 ml of an eluent containing biotinylated peptide are collected and lyophilized. To evaluate the ratio of covalently immobilized biotin molecules to the Y939 peptide, the amounts of the Y939-immobilized biotin and the Y939 peptide in the eluent are determined as follows. After lyophilization, the biotin-Y939 conjugate is dissolved in 1.0 ml of a PBS buffer and its absorbance at 280 nm is measured. Assuming that the observed absorbance is due to a tyrosine residue in a Y939 peptide, total amount of Y939 pep-

tide is obtained from the absorbance and molar absorptivity for tyrosine of $1.34 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The amount of Y939-conjugated D-biotin is evaluated by a spectrophotometric assay method.

Immobilization of the Y939 Peptide on a 96-Well Microtiter Plate

All 96 wells of a microtiter plate are incubated with 150 μ l each of avidin solution (0.1 mg/ml avidin in PBS buffer) over 18 h at 4°C. After the immobilization of avidin on the well, each well of the plate is washed with a PBS-T buffer solution (0.05% Tween 20 in PBS buffer). To fill up space between the immobilized avidin molecules with BSA molecules, a 200- μ l portion of a BSA solution (1.0% BSA in PBS buffer) is added to each well and the plate is left for 4 h at 4°C. The BSA solution is discarded, and each well is washed four times with the PBS-T solution. After washing with the PBS-T solution, 150 μ l of biotin-Y939 conjugates (1.0 μ g/ml in the PBS-T solution) is added to each well and biotin-avidin complexation is carried out for 1 h at 4°C. An excess of the conjugates is discarded, and the well is washed four times with the PBS-T solution.

Kinase Reaction

A sample solution is prepared in a microtube, consisting of 0.05% Triton X-100, 50 μ M ATP, 1.7 mM MnCl₂, a given concentration of IR, 50 mM HEPES/NaOH (pH 7.4), and each concentration of insulin. The sample solution is incubated in a microtube at 25°C for 10 min, and 150- μ l portion of the sample solution thus prepared is introduced in a given well. For obtaining a background absorbance, the sample solution without insulin is also introduced in one of the wells. The plate is shaken on a mixer for 1 h at 37°C to react the IR immunocomplex with the Y939 peptide immobilized on the well surface. The plate is washed three times with the PBS-T solution and then two times with a TBS solution (20 mM NaCl, 3.0 mM KCl, and 20 mM Tris/HCl, pH 7.4).

Enzymatic Reaction and Absorptiometric Assay

After dilution of monoclonal anti-phosphotyrosine antibody labeled with horseradish peroxidase in the TBS solution to its final concentration of 0.5 μ g/ml, 150- μ l of the solution is added into each well. After standing for 2 h at room temperature without shaking, the plate is washed six times with a TBS-T (0.05% Tween 20 in the TBS buffer) solution. To evaluate the amount of the antibody specifically bound to the phosphorylated Y939 peptide, 175 μ l of a DA-64 solution

(0.4 mg/mL of DA-64 in 2.0% H₂O₂, 50 mM citric acid, and 100 mM Na₂HPO₄) is added to each well and is incubated with shaking for 1 h at 37°C in the dark. After the enzymatic reaction, the DA-64 solution is subjected to absorbance measurement. A 150- μ l portion of the DA-64 solution is diluted with 1.0 ml of Milli-Q water, and the absorbance of the solution in a 1.0-ml cuvette is measured at 727 nm against water with a spectrophotometer.

EVALUATION

For obtaining net insulin-dependent signals (absorbance), a background absorbance obtained by introducing a buffer solution without insulin is subtracted from the observed absorbance in the presence of a given concentration of insulin. Changes in absolute value of this net absorbance are not negligible from one microtiter plate to another, seemingly because of unexpected alteration in the experimental conditions such as concentration of antibody and/or a decrease in activities for IR kinase. In order for the net absorbances obtained from one microtiter plate to be comparable to those obtained from another, the measured net absorbance for a given concentration of insulin is normalized against absorbance for 1.0 μ M insulin in terms of $B/B_{\max} \times 100$, where B is the absorbance for each concentration of insulin and B_{\max} that for 1.0 μ M insulin.

K.6.3.1.3.2.3

Detection by TRF

PURPOSE AND RATIONALE

A time-resolved fluorescent assay using Wallac's DELFIA system (DELFLIA assay) has been developed to monitor changes in IR tyrosine phosphorylation. Usually phosphorylation levels are analyzed by measuring the incorporation of radioactive phosphate (Tornqvist and Avruch 1988), by immunoblotting, or by ELISA (Krutzfeldt et al. 1999) using phosphotyrosine antibodies (see K.6.3.1.3). These methods suffer from various drawbacks including the use of radioisotopes, tedious and limited sample analysis with respect to immunoblotting, and low dynamic range, in particular in colorimetric assays using ELISA. In contrast, the DELFLIA assay using time-resolved fluorometry is a highly sensitive, non-radioactive assay performed in microtiter plates amenable to automation. A format similar to that of the DELFLIA assay has been used by Okada and coworkers (1998) to measure the effect of sodium orthovanadate on IR autophosphorylation of erythrocytes from normal and diabetic patients.

PROCEDURE

Cell Culture

Chinese hamster ovary (CHO) cells overexpressing the hIR (CHO-hIR) are maintained in DMEM, 10% (by vol.) FBS and 1% (by vol.) penicillin-streptomycin at 37°C under 5% CO₂. Before treatment with insulin/compounds/drug candidates, cells are serum-starved for at least 14 h by incubation in DMEM, 0.5% (w/v) BSA, 1% penicillin-streptomycin.

In Vitro Phosphorylation of the IR

Wheat germ agglutinin-purified hIR is prepared from total membranes of CHO-hIR as described (K.6.3.1.1.2 and K.6.3.1.3.1) and then phosphorylated by incubating 700 μ g of hIR with 50 mM HEPES (pH 7.4), 10 mM MnCl₂, 0.1% (by vol.) Triton X-100, 1 μ M insulin, 0.5 mM ATP, and 100 μ M sodium orthovanadate for 1 h at room temperature. The phosphorylated hIR is purified by passing the solution over a PD-10 column (Pharmacia) in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 100 μ M sodium orthovanadate and concentrated by centrifugation in a 2-ml Amicon concentrator (30MWCO).

Biotinylation of Anti-IR Antibody

Anti-IR antibody Ab-3 (800 μ l of 1.3 mg/ml, Upstate Biotechnology) is biotinylated by incubating overnight at 4°C with 60 μ M of freshly prepared Sulf-NHS-LC-biotin (Pierce) dissolved in 4 mM bicarbonate (pH 7.8), 35 mM NaCl, 1% (by vol.) glycerol (final vol. 1 ml). Buffer exchange (PBS) and excess biotin removal are facilitated by diluting and concentrating in centricon units (30MWCO). The level of biotin incorporation is quantitated by comparing to a standard curve constructed using the biotin-induced displacement of avidin from the dye 2-(4-hydroxybenzene azo benzoic acid (HABA; Pierce; absorption of HABA-avidin 500 nm) according to the manufacturer's instructions. The typical yield is between 4 and 10 moles of biotin per mole of protein.

Preparation of Phosphorylated IR from Cells

Cells are grown in 6-well plates until ~60% confluency and serum-starved overnight. The cells are washed with DMEM, incubated in 2 ml of the same medium, and treated with various concentrations of insulin/compounds/drug candidates for 5–10 min. Following the treatments the medium is removed and the cells are immediately frozen in liquid nitrogen and then stored at –80°C until cell lysis. Cells are lysed in 200 μ l of lysis buffer (50 mM Tris, pH 7.4,

150 mM NaCl, 1% Triton X-100, 1 mM pyrophosphate, 100 μ M pervanadate, “Complete” protease inhibitor tablet). The cells are gently scraped from the dishes and centrifuged (14,000 rpm, 15 min, 4°C, Eppendorf centrifuge). The supernatants are used in the DELFIA assay.

Quantitation of Receptor Phosphorylation

All incubations of the DELFIA assay are carried out at room temperature with agitation in a final volume of 200 μ l of DELFIA assay buffer unless otherwise stated. Streptavidin-coated plates are washed with DELFIA wash buffer for 15 min. After removal of wash buffer, anti-IR–biotin or anti-EGFR–biotin (100 ng/well) is added for 30 min. The plate is washed three times with wash buffer and cell lysates are added and the plate is incubated for 1 h. The plate is again washed three times with wash buffer, europium-labeled anti-phosphotyrosine antibody (100 ng/well) is added, and the plate is incubated for 1 h. Following extensive washing (at least five times), 180 μ l of enhancement solution is added. The plate is shaken vigorously for 30 min and read in a microtiter plate reader using an europium protocol (excitation at 340 nm, emission at 615 nm, delay 400 μ s, 1000 μ s/cycle).

Optimization of Conditions

This time-resolved fluorescent (TRF) assay for measuring tyrosine-phosphorylated proteins in cells has been optimized by Waddleton and coworkers (2002). For this, biotinylated anti-IR antibody is used to capture both phosphorylated and non-phosphorylated IR from cells and for immobilization to the streptavidin-coated plate. Europium-labeled anti-phosphotyrosine antibody is used to bind only the phosphorylated IR. Time-resolved fluorometry of europium is then used to measure the level of phosphorylated IR. To establish conditions for the DELFIA assay, *in vitro* phosphorylated IR partially purified from CHO hIR cells is used. The optimum concentrations of both the capture antibody (anti-IR antibody) and the anti-phosphotyrosine antibody is typically \sim 100 ng. Using *in vitro* phosphorylated partially purified CHO hIR (0.13–0.52 μ g/ml) the Eu signal typically reaches three orders of magnitude and remains linear to up to \sim 800,000 Eu counts. The minimal amount of phosphorylated IR necessary for a reliable signal is \sim 0.1 pmole (in 200 μ l). The signal is specific for the phosphorylated form of IR since non-phosphorylated IR tested at similar concentrations does not produce a significant increase in background signal. Establishing the optimal conditions for the DELFIA assay it has been demonstrated that the

phosphorylation status of the IR does not affect the ability of the capture antibody to immunoprecipitate IR from cell lysates.

EVALUATION

Stimulation of the cells with insulin followed by measurement of IR phosphorylation by DELFIA resulted in an EC₅₀ of \sim 25 nM. This is similar to that reported by Biazzo-Ashnault and coworkers (2001) who used a FRET assay to measure IR tyrosine kinase activity by following phosphorylation of a synthetic peptide. Using the non-specific, protein tyrosine phosphatase (PTP) inhibitor, pervanadate, a 10-fold increase in basal IR phosphorylation is typically detected by the DELFIA assay, whereas analysis of the same samples by immunoblotting reveals a 3-fold increase, only. This difference is likely due to enhanced sensitivity of the DELFIA assay, particularly in the lower range where quantitation of scanned immunoblots is less accurate. Pervanadate results in a 50% increase in insulin-induced IR phosphorylation compared to treatment with ligand alone (10 nM), as measured by both DELFIA and immunoblotting. Comparing the well-established technique of immunoblotting with anti-phosphotyrosine antibodies to the DELFIA assay, similar results are usually obtained. In addition, Waddleton and coworkers (2002) demonstrated that this assay can be used to monitor IR phosphorylation in a normal (i. e. IR non-overexpressing) cell line and in response to phosphatase inhibitors. They also show the rapid and easy adaptation of the Delphia format assay to monitor changes in the phosphorylation status of other cellular proteins. These findings provide strong evidence that changes in the phosphorylation status of the IR in response to PTP inhibitors and most likely insulin-like compounds/drug candidates can be monitored using the DELFIA assay.

K.6.3.1.3.2.4

Yeast-Based Method

GENERAL CONSIDERATIONS

A number of mammalian cell-based assays have been developed in order to analyze IR kinase activity in response to compounds/candidate drugs in a cellular environment that most closely resembles the natural physiological conditions (see K.6.3.1.3.2 and K.6.3.1.2; Sims and Allbritton 2003, Sato et al. 2002). However, mammalian cell-based assays are expensive and time-consuming. Moreover, the effects of redundant processes on the measured output can be difficult to control and to distinguish from the effects that are specific for the IR. The yeast *Saccharomyces cere-*

visiae represents an inexpensive and rapid alternative for measuring the activity of the IR in a heterologous, yet eukaryotic environment. The fact that yeast does not have any endogenous RTKs and no *bona fide* non-receptor tyrosine kinases offers the advantage of a null background for the expression of the mammalian IR and for the measurement of the effects of compounds/drug candidates on the specific target (Barberis 2002, Gunde 2004, Hughes 2002). A yeast growth selection system for monitoring mammalian RTK activities and for detecting inhibition by specific inhibitors, which is based on the Ras-recruitment system (RRS) developed by Aronheim and collaborators has been introduced recently (Broder et al. 1998, Aronheim et al. 1994, 1998) and further modified and improved by Gunde and Barberis (2005). In particular, it may be useful for the throughput-screening for insulin-like compounds/drug candidates acting directly at the IR.

PURPOSE AND RATIONALE

Cdc25p, the yeast homolog of mammalian son of sevenless (Sos), is a Ras guanyl nucleotide exchange factor (Ras-GEF) functioning upstream of the Ras proteins in *S. cerevisiae*. The yeast temperature sensitive *cdc25-2* strain cannot grow at 37°C, because the mutant Cdc25 protein carried by this strain is unable to activate the endogenous Ras protein at this non-permissive temperature. Aronheim and coworkers (1994, 1998) have shown that membrane targeting of the mammalian Sos is sufficient to activate Ras leading to complementation of the *cdc25-2* temperature sensitive allele. The yeast growth selection system for monitoring human IR is based on the RRS.

PROCEDURE

Yeast Strains and Yeast Spotting Assay

Cells from the yeast strain *cdc25-2* (Mata α , ade5, *cdc25-2*, his7, lys2, met10, trp1, ura3-52, leu2) transformed with the hIR are plated on 2% glucose agar plates. Four different colonies of each transformation are picked and spotted onto two sets of galactose plates (3% galactose, 2% raffinose, and 2% glycerol) that are incubated at 25°C or 37°C. For this spotting assay, the yeast suspensions are not adjusted to the same concentration prior to spotting, whereas for the serial dilutions, the yeast cultures are adjusted to the same concentration. Plates incubated at 25°C are scanned after 4 days, whereas plates incubated at 37°C are scanned after 7 days. For serial dilutions, exponentially growing cultures are serially diluted in sterile water starting with a concentration of 4×10^7 cells/ml. 5 μ l of each dilution are spotted onto two sets of galactose plates, cor-

responding to about 100,000, 20,000, 4000, 800, 160, and 32 cells *per spot*. Plates are incubated at 25°C and 37°C and scanned after 4 and 11 days, respectively.

Preparation of Yeast Extracts and Immunoblot Analysis

Exponentially growing *S. cerevisiae* cultures expressing the hIR are diluted to a final absorbance at 600 nm (A_{600}) of 0.5 in 4 ml of Ura/-Leu/-Trp dropout medium containing 2% glucose and are grown for 4 h. Cells are harvested by centrifugation, and whole cell extracts are prepared as described (Kaiser et al. 1994). Samples of whole cell extracts subjected to SDS-PAGE and blotted on nitrocellulose membranes following standard protocols.

Growth

Exponentially growing cultures expressing hIR are washed once with water and diluted to a final A_{600} of 0.1 ml of Ura/-Leu/-Trp dropout medium containing 3% galactose, 2% raffinose, and 2% glycerol. As a control, cells expressing M-Grb2-Ras(Q61L) Δ F are similarly grown, washed, and diluted to a final A_{600} of 0.03. 150 μ l of the diluted cells are added to 96-well plates containing 1.5 μ l of the compounds/drug candidates at the desired concentrations (1% final DMSO concentration). The plates are incubated at 37°C, and growth at the indicated time points is quantified by measuring A_{595} with a microtiter plate reader.

EVALUATION

As alternatives to this yeast Sos-recruitment system (RRS) to monitor protein-protein interactions at the plasma membrane, the reverse RRS for the identification of protein-protein interactions with integral membrane proteins (Köhler and Muller 2003), the split-ubiquitin system (Hubsman et al. 2001), and the SCINEX-P system (Stagljar et al. 2001) have been developed during the last decade. The split-ubiquitin system has been used to detect novel proteins that are associated with the mammalian ErbB3 RTK (Thaminy et al. 2003). Ehrhard and coworkers (2000) developed a method that uses the yeast G-protein signaling pathway as readout to select for protein-protein interactions. This assay has been validated with known binding partners, such as the RTK fibroblast growth factor receptor 3 (FGFR3) and SNT-1, that constitutively binds to the FGFR3 intracellular domain. The latter two methods are feasible for detection of interactions between an integral membrane protein and a soluble cytoplasmic protein, but, in contrast to the direct RTK assays, the binding of cytoplasmic proteins to the RTKs is independent of receptor activity.

Furthermore, Köhler and Muller (2003) recently applied an adaptation of the RRS to the analysis of interactions between two membrane-associated proteins, namely the myristylated and phosphorylated EGFR intracellular domain and a myristylated Grb2-Ras fusion protein. Due to the fact that yeast cells do not have endogenous mammalian-type tyrosine kinases, the yeast-based growth selection system offers the advantage of a null background eukaryotic organism for expression of these mammalian membrane-bound kinases, such as the IR. This assay may be of value in throughput screenings for the identification of cell-active activators (and inhibitors) of RTKs.

K.6.3.2

Phosphorylation of Signaling Components by Insulin and Insulin-Like Stimuli

GENERAL CONSIDERATIONS

Protein kinases (PK) and phosphatases (PP) have been implicated in a variety of cellular processes such as proliferation, differentiation, metabolism and apoptosis. Over one-third of the proteins in the human proteome are phosphoproteins and the family of PK and PP represents up to 5% of the human genome. These enzymes may cause an increase in or suppression of the activity of other enzymes, receptors, regulatory proteins or transcription factors, mark proteins for destruction, allow proteins to move from one subcellular compartment to another, or enhance or impede protein-protein interactions. In many cases these components and processes are embedded in various signaling pathways, such as the insulin signal transduction cascades. Thus, any change in the level, activity, or localization of PK and PP has a major impact on the regulation of these processes. Not surprisingly, the dysregulation of protein phosphorylation has been associated with diabetes.

There are estimated to be over 500 different PK in the human proteome that catalyze the transfer of phosphate from ATP to serine, threonine, and tyrosine. PK can be divided into two broad classes, serine/threonine and tyrosine kinases, although a few PK are able to phosphorylate both types of amino acid residues (dual-specificity kinases). Because of the key role that PK play in cellular functions, they have become one of the most important drug targets. As such, protein phosphorylation is in the focus of much interest for the development of compounds to selectively regulate the activity of diabetes-related PK. Thus, there is a need for assay systems that monitor the activity of these enzymes under a variety of experimental conditions and

for the development of selective inhibitors or activators of these enzymes for a variety of therapeutic applications, among them metabolic diseases, such as diabetes.

PURPOSE AND RATIONALE

One strategy is based on the identification of selective novel inhibitors of PK, which have been implicated with interference of insulin signal transduction *via* serine phosphorylation of key signaling components, such as IRS-1 (see K.6.3 and K.6.3.4), thereby blocking activation of downstream components, such as PI3-K. These compounds/drug candidates should act as insulin-sensitizers bypassing the insulin resistance in muscle and liver tissues of type II diabetic patients. The other strategy relies on the identification of activators of PK, which function as positive elements in the insulin signal transduction cascade, such as the IR. These compounds/drug candidates may induce insulin-like signals in muscle and liver tissues of type II diabetic patients. In any case, the effect of large libraries of compounds on the phosphorylation of specific insulin signaling components has to be analyzed in a rapid, reliable and sensitive fashion.

PROCEDURE

Numerous methods have been designed to measure PK activity. Early methods require physical separation of substrate protein or peptide from its phosphorylated product. One method is to use radiolabeled ATP that allows the incorporated phosphate to be detected after the capture of the protein/peptide substrate by binding to phosphocellulose membranes (Witt and Roskoski 1975). This type of PK assay requires multiple steps and the use of radioactivity with its accompanying hazards and costs. Previously, a very selective and highly sensitive assay for various protein kinases was developed that is based on the use of biotinylated peptide substrates and [γ - 32 P]ATP. This assay is fast, quantitative, and does not require any alteration of the peptide consensus sequence of the optimal PK substrate, which is routinely done with phosphocellulose membrane-based kinase assays. Although this assay system offers many advantages such as specificity, sensitivity, and convenience, it suffers from the fact that it is not homogeneous, i. e., it requires transfer of reaction products to a filter plate, and it is radioactive. Others have developed assays for measuring the enzyme activity of PK that are homogeneous and increase throughput for measuring the PK activity of many samples. Some of these are radioactivity-based assays, such as the scintillation proximity assays (SPA) or fluorescence-based

assays, such as time-resolved fluorescence resonance energy transfer (FRET) and fluorescence polarization (FP). The SPA and flashplate assay have been used as a high-throughput homogeneous assay of PK activity that does not require an antibody (Braunwalder et al. 1996, Antonsson et al. 1999), but still has some disadvantages. Although the signal-to-noise ratio (S/N) is high for the SPA, it uses radioactivity ($[\gamma\text{-}^{33}\text{P}]\text{ATP}$), and its efficiency is dependent on the energy of the isotope used ($[\gamma\text{-}^{33}\text{P}]\text{ATP}$ is preferred over $[\alpha\text{-}^{32}\text{P}]\text{ATP}$). It also requires centrifugation or washing steps. Homogeneous non-radioactive assay formats measuring PK activity, that include FP and FRET, have been developed to eliminate the disadvantages of radioactivity and washing steps (Seethala and Menzel 1997, Park et al. 1999). FP is a ratiometric technique that reflects the rotational motion of a fluorescent molecule in solution with the rotational velocity dependent on the apparent molecular size (Pope et al. 1999). Although FRET and FP are non-radioactive, their signal-to-noise ratios are low and they rely on the availability of very high affinity and selective antibodies. This represents a major drawback of FRET, its reliability on the dual-label format in which an anti-phosphopeptide antibody is labeled with a fluorescence donor and is brought into close proximity to the peptide conjugated to a fluorescence acceptor, and only when these two molecules are within close proximity does transfer occur. FP on the other hand, uses only one labeled fluorescent molecule and it is ratiometric and thus minimizes interference by sample quenching. The disadvantages, however, are the very low S/N ratio that in some cases is less than 1.5, depending on the assay. The optimization of the assay may be difficult to attain because one cannot easily predict the appropriate location and spatial configuration for the fluorescent label. A major disadvantage of both FP and FRET assays is that they are dependent on the availability of a phospho-specific antibody. Another drawback to the use of antibodies is that their high specificity for a given phosphopeptide often prevents the same antibody from being used with different substrates. Anti-phosphotyrosine-specific antibodies are broadly reactive since they are relatively insensitive to the surrounding amino acid context. In contrast, anti-phosphoserine-/phosphothreonine-specific antibodies are highly dependent on the amino acid sequence surrounding the phosphorylated residue and thus tend to only bind a limited diversity of phosphorylated sites (Wu et al. 2000). In general, a new antibody must be generated for each different substrate, which can be both time consuming and expensive. There are also

ELISA-based assays for PK activity, but these also require multiple steps in addition to being dependent on availability of a phospho-specific antibody to capture product (Angeles et al. 1996).

Several antibody-free non-radioactive assays for PK activity have been reported. One of these is based on the differential electrophoretic mobility of the fluorescent peptide substrate and its phosphorylated product using capillary electrophoresis and is compatible with throughput formats. This type of assay requires specialized high-throughput capillary electrophoresis setup and equipment. Another assay method replaces ATP in the reaction with ATP γ S leading to the formation of a thiophosphorylated peptide product (Jeong and Nikiforov 1999). This product is then chemically biotinylated by a sulfur-specific biotinylation reagent. Streptavidin is then added to the fluorescently tagged peptide and an FP measurement is taken to measure the amount of product formed. The potential disadvantages of this assay include long incubation times and altered kinetics for some PK due to the ATP γ S. Another antibody-free FP assay of PK activity that has been reported uses polyarginine to discriminate fluorescently labeled phosphorylated and non-phosphorylated peptides based on charge interaction of the phosphate group and the positively charged high molecular weight polyarginine (see K.6.3.2.3; Coffin et al. 2000, Simeonov et al. 2002). Another potential antibody-free assay of PK activity is the so-called IMAP assay that employs trivalent metal ions immobilized on nanoparticles to bind phosphate groups on molecules and thus increase their FP values (Huang et al. 2002). This approach is similar in concept to immobilized metal affinity chromatography (IMAC) used to specifically isolate phosphopeptides from mixtures containing both phosphorylated and non-phosphorylated peptides (Anderson and Porath 1986). Finally, the development of microchip instruments has led to an additional approach for measuring the PK activity (Cohen et al. 1999). In the microfluidic assay of PK activity, fluorophore-labeled peptide substrate and product are separated based on a difference in their charge-to-mass ratio. Separation of product and substrate is achieved by electrophoresis through an electric field, followed by quantitation of the individual fluorescent peaks. The currently most important and widely used PK assay technologies as well as novel ones with interesting future applications are described briefly.

K.6.3.2.1**Scintillation Proximity Assay (SPA)**

For the scintillation proximity assay (SPA), the final assay conditions are 50 mM Tris (pH 8.0), 10 mM MgCl₂, 0.01% Triton X-100, 1 mM DTT, 1 or 3.75 μM ATP, 0.375 μM biotinylated peptide substrate, 160 ng/reaction kinase, 10 μM compound (in 10 mM DMSO, diluted 1:100 at least) and 0.125 μCi/reaction of [³³P]ATP. The 40-μl reactions are incubated at room temperature for 4 h. A stop reagent (40 μl per well) containing 0.2 mg streptavidin-coated SPA beads (Amersham), 40 mM EDTA, and 74% CsCl is added to the reaction. After sealing the plates and incubating for 2 h at room temperature, the plates are read on a microtiter plate TopCount scintillation counter.

K.6.3.2.2**Fluorescence Polarization (FP) Assay****PURPOSE AND RATIONALE**

FP is a solution-based non-radioactive homogeneous assay that requires no separation of components for the analysis and in many but not all cases (see below) depends on the availability of appropriate phosphosite-specific antibodies. FP, first described in 1926, depends on the differing rotational properties of small (<10,000 Da) vs. large (> 100,000 Da) molecules. Fluorescent molecules excited by a plane of polarized light emit light in that same plane if the molecules do not move or rotate during the course of the excited state (1–5 ns). If the fluorescent molecules change position during their existence in the excited state, the polarized light will be emitted in a plane different with respect to the excitation plane. Large molecules such as proteins (or small molecules bound to proteins) do not rotate or move significantly during the lifetime of the excited state. Therefore, they do not become depolarized during the course of an FP experiment. Small molecules, such as peptides, tumble more rapidly and thus become depolarized during the course of the FP experiment. This change in polarization can be detected by monitoring the amount of fluorescence in the vertical vs. the horizontal plane following excitation in the vertical plane. Mathematically this can be expressed as polarization value (P) = $(I_V - I_H) / (I_V + I_H)$, where I_V and I_H are the intensities of the vertical and horizontal components of the emission light, respectively. FP is a dimensionless entity and is not dependent on the intensity of the emitted light or on the concentration of the fluorophore. This is a fundamental advantage of FP, in that each sample has an internal calibration that thus

limits variations in intensity due to source fluctuation, fluorescence quenching by contaminants or inhibitors, or scattering caused by turbidity. The major limitation of FP lies in the limited sensitivity and signal-to-noise ratio, requiring specialized fluorimeters.

PROCEDURE

Most commercially available FP-based assays of PK activity require an anti-phospho-specific antibody. There are several disadvantages to the use of antibodies in FP assays. Although anti-phosphotyrosine-specific antibodies are common and relatively amino acid context independent, they are expensive. Anti-phosphoserine- or phosphothreonine-specific antibodies can be difficult and costly to produce and are sensitive to amino acid sequence surrounding the phosphorylation site and hence cannot be used for all serine/threonine peptide substrates (see K.6.3.2 and K.6.3.3.4). Unlike most other homogeneous fluorescent assays of PK activity, reagents required for the zinc FP assay are simply a fluorescein-labeled peptide and commonly used, inexpensive laboratory reagents, with no antibody required.

Modifications of the method have been established which are based on the use of Zinc (Scott and Carpenter 2003, polyarginine (Coffin et al. 2000) and competitive mode of assaying (Seethala and Menzel 1998, Turek et al. 2001, Kristjansdottir and Rudolph 2003).

K.6.3.2.3**Fluorescence-Based Assay****PURPOSE AND RATIONALE**

To address the various drawbacks of existing assays, a novel, robust system to measure the activity of several PK has been developed. This assay is rapid, can be completed in less than 2 h, and can be carried out in multiwell plate formats such as 96- or 384-well plates. The signal-to-noise ratio is very high, and unlike FRET or FP, it uses a simple measure of fluorescence intensity. The assay also does not rely on the availability of high-affinity and selective antibodies, as is the case with other existing technologies. Finally, the assay is easily adapted to robotic systems for drug discovery programs.

PROCEDURE

It has been reported that phosphorylation of peptides on serine (Murray et al. 1996) or tyrosine (Dass and Mahalakshmi 1996) decreases the proteolytic activity of proteases. Thus, phosphorylation of peptides by PK

can be monitored by the increase in resistance to protease cleavage of the phosphorylated product. The difference in cleavage rate of the peptide in the absence and presence of phosphorylation can be used as a measure of the PK activity. It is noteworthy that the activity of PP can also be determined using a similar approach. The cleavage rate can be monitored by HPLC separation of the peptides before and after phosphorylation. However, this approach is tedious, time-consuming, requires large amounts of substrates and PK/PP, and is not practical for a large number of samples. To develop a PK assay based on this principle that is sensitive, simple, and scalable to high-throughput formats, Kupcho and coworkers (2003) introduced a fluorescence technology based on the use of Rhodamine 110 (R110)-modified peptide substrates.

Free R110 is a fluorescent molecule but when it is covalently linked *via* both its amino groups in a bisamide form both its visible absorption and its fluorescence are suppressed. Upon enzymatic cleavage, the non-fluorescent bisamide substrate is converted in a two-step process, first to the fluorescent monoamide and then to the fluorescent monoamide and then to the even more fluorescent free R110. Both of these hydrolysis products exhibit spectral properties similar to those of fluorescein, with peak excitation and emission wavelengths of 496 and 520 nm, respectively. When the peptide substrate is not phosphorylated, it can be digested by aminopeptidase, which releases free R110 and thus enhances fluorescence at 520 nm. Phosphorylation of the peptide slows down the proteolytic cleavage of the peptide and thus minimal change is observed in the fluorescence. Using the appropriate consensus sequence for the peptide substrates, which have to be coupled to R110 in a bisamide form according to the protocol from Kupcho and coworkers (2003), the activity of a multitude of different PK can be monitored.

K.6.3.2.4

Capillary Electrophoresis-Based Assay

PURPOSE AND RATIONALE

Separation of non-phosphorylated substrate from the phosphorylated product can be accomplished by capillary zone electrophoresis. Dawson and coworkers (1994). Although the separations are very efficient, each separation run takes about 10–15 min, making this approach too slow for the screening of large chemical libraries. The microchip is uniquely suited for the development of PK assays based on separation of substrate and product. Using computer control, it is possible to dispense nanoliter volumes of reagent solu-

tions into the channel network. Enzymatic phosphorylation of the substrate takes place while the mixture is flowing through a reaction channel. An aliquot of the mixture is then injected into a separation channel where substrates and product are electrophoretically separated. The separation requires only a fraction of the time needed in traditional capillary electrophoresis. Cohen and coworkers (1999) presented the successful development of a microchip-based assay for a model PK, PKA, activity. This assay format may be applied to a wide range of different PK, for which fluorogenic substrates do not exist but where the outcome of the enzymatic reaction can be assessed by the separation of substrate from product.

PROCEDURE

The assay buffer for on-chip experiments using PKA is 100 mM HEPES/KOH (pH 7.5), 5 mM MgCl₂, 100 μM ATP, 50 μM cAMP, and 1 M NDSB-195 (dimethylethylammonium propane sulfate) and is filtered through a 0.22-μm disposable Millipore syringe filter. The design and fabrication of the chips is given in the published report (Cohen et al. 1999). Prior to use, the chips are thoroughly cleaned with deionized water, 2-propanol, a filtered solution of 1 N NaOH for 15–30 min, rinsed with water and finally filled with assay buffer. To run experiments, the microchips are filled with the required reagents and placed in a specially constructed holder on the stage of an epifluorescent inverted microscope (480/40-nm excitation filter; 535/40-nm emission filter). A lid containing Pt electrodes is placed over the chip. Upon lid closure, the electrodes are inserted into the wells of the chip. These electrodes are connected to the high-voltage controller. Currents and voltages are applied to platinum wire electrodes immersed in reagent wells on the microchip.

EVALUATION

The capillary electrophoresis-based assay format may replace other methods of analysis of PK, namely radiolabel assays and other more cumbersome heterogeneous formats, the true appeal of the microchip is its potential for application to high-throughput screening. Significant reductions in assay time and reagent consumption will offset the expense by the need for instrumentation and software development. Optimization of channel dimension for the direct introduction of compounds/drug candidates from a microtiter plate into the on-chip reagent stream of substrate and enzyme as well as of reagent concentrations as methods to manipulate on-chip incubation times for enzymes of varying turnover rates have meanwhile been achieved.

K.6.3.3**Dephosphorylation of Insulin and Insulin-Like Signaling Components****GENERAL CONSIDERATIONS**

The PTP (protein tyrosine phosphatases) are a structurally diverse family of both receptor-like and non-transmembrane hydrolases that have been implicated in the control of numerous physiological processes, including growth, differentiation and metabolism (Johnson et al. 2002). Approximately 75 PTPs have been identified to date. These enzymes are characterized by the presence of a conserved catalytic domain of 240 residues which contains the unique signature motif, C(X₅)R, that defines this enzyme superfamily (Andersen et al. 2001, Alonso et al. 2004). The structural diversity is manifested by the variability in non-catalytic sequences fused to the amino- or carboxy-terminus of the catalytic domain. These non-catalytic segments frequently serve a regulatory function, including ligand binding for receptor PTPs and targeting of cytoplasmic PTPs to defined subcellular locations. Despite variations in primary structure and differences in substrate specificity, key structural features in the catalytic site and mechanism are conserved among all members of the PTP superfamily (Barford et al. 1994 and 1998, Zhang 2003). Although great progress has been made in illustrating the, somewhat unexpected, structural diversity within this large enzyme family, relatively little is known of the physiological function of individual PTP. Presumably this structural diversity reflects a broad range of functions for the PTP family *in vivo*. However, it is clear on the basis of the role of protein tyrosine phosphorylation in insulin signal transduction that PTP play important roles in the down-regulation of the insulin signal either for the physiological termination of insulin action after removal of insulin from the circulation or during the pathogenesis of type II diabetes in course of the development of insulin resistance in peripheral tissues. In particular, the potential role of PTP1B as a negative regulator of insulin action, causally related to the pathogenesis of insulin resistance and type II diabetes has been suggested by a number of studies using knock-out animals and cultured cells with ectopic overexpression and inhibition by small molecules or antisense oligonucleotides (Johnson et al. 2002, Elchebly et al. 1999). Consequently, PTP, in general, and PTP1B, in particular, have been recognized as attractive targets for the identification of insulin-like/sensitizing drugs for future anti-diabetic therapy.

PURPOSE AND RATIONALE

Considerable work has been directed at identifying PTP that dephosphorylate IR as possible targets for insulin-like compounds/drug candidates. Several receptor PTP (e. g. PTP- α , LAR) and non-receptor PTP (PTEN, PTP1B) have been considered candidates based on studies of their overexpression, substrate-trapping mutants, mouse knockout models, and other approaches (Cheng et al. 2002, Tonks 2003, Bourdeau et al. 2005). Since the inhibition of PTP1B causes prolonged and increased IR phosphorylation, it is a potential therapeutic target in the treatment of type II diabetes and insulin resistance.

As interest in understanding PTP1B and its role in the regulation of insulin signal transduction and pathogenesis of type II diabetes grows, the accurate measurement of tyrosine phosphorylation on proteins becomes increasingly important. Therefore to aid in the identification of selective, potent PTP1B inhibitors novel assays have recently been designed to follow changes in tyrosine phosphorylation of the IR and of additional so far unknown substrate proteins as well as for the identification of novel PTP1B substrate proteins in normal and insulin-resistant muscle, adipose and hepatoma cell lines which all express functional PTP1B and IR.

In addition to PP, lipid phosphatases have also been implicated as negative regulators of insulin signaling and action, in general, and insulin-stimulated GLUT4 translocation, in particular. In addition to positive regulators of GLUT4 translocation, negative regulators have also been studied intensely because they might provide opportunities for pharmacological interventions. For example, the endogenous attenuation of PI3-K signaling occurs through the dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P₃]. Two phosphoinositide phosphatases, SHIP2 (type-II SH2-domain-containing inositol 5'-phosphatase) and PTEN (phosphatase and tensin homolog deleted on chromosome ten), have been implicated in negatively regulating insulin signaling (Jiang and Zhang, 2002). SHIP2 removes the 5' phosphate from PtdIns(3,4,5)P₃ to generate phosphatidylinositol-3,4-bisphosphate [PtdIns(3,4)P₂]. Although the *Ship2* KO mice die shortly after birth and are severely hypoglycemic, heterozygous animals show improved glucose tolerance and enhanced insulin sensitivity (Clement et al. 2001).

In contrast to SHIP2, PTEN is a 3'-specific PtdIns(3,4,5)P₃ phosphatase that generates PtdIns(4,5)P₂. Overexpression of PTEN prevents the accumulation of PtdIns(3,4,5)P₃ and also inhibits

insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes (Nakashima et al. 2000). Moreover, the genetic ablation of PTEN in adipose tissue of mice has recently been shown to enhance insulin sensitivity and resistance to pharmacologically induced diabetes (Kurlawalla-Martinez et al. 2005).

K.6.3.3.1

Protein Tyrosine Phosphatase (PTP) Activity Measurement Using DIFMUP

PURPOSE AND RATIONALE

A variety of substrates have been developed for measurement of the hydrolytic activity of PTP with the rather unspecific *para*-nitrophenylphosphate (PNPP) and specific phosphotyrosine-containing peptides being most widely used. These assays have significantly improved our understanding of substrate specificity and catalytic mechanism of PTP. However, their use for routine inhibitor screening is rather limited due to a variety of reasons, in particular, the requirement for synthesis of a phosphopeptide, low sensitivity in detection, susceptibility to color interference, and discontinuous nature (i. e. termination of the reaction by addition of acidic malachite green reagent or alkaline solution; Zhang and Dixon 1994, Zhang et al. 1993). A number of fluorogenic compounds used for the analysis and characterization of PTP have been described. Recently, Huang and coworkers (1999) and Wang and coworkers (1999) described the development of fluorescein monosulfate monophosphate as a dual fluorogenic substrate for both phosphoserine/threonine phosphatases and PTP. The similarity in their reaction mechanisms seems to be the reason that phosphatases from the different families can use these small non-peptidic substrates, albeit with pronounced differences in their catalytic efficiencies. Wang and coworkers (1999) described the development of fluorescein monosulfate monophosphate and their use as sensitive fluorogenic substrates for several PTP. The demonstrated catalytic efficiencies which are comparable to those of phosphopeptide substrates favor their use in alternative more sensitive assay designs. Recently, another fluorogenic substrate, 6,8-difluoro-4-methylumbiliferyl phosphate (DIFMUP) was found to be appropriate for measurement of PTP activity (Welte et al. 2003). This substrate has initially been developed for phosphoserine/threonine phosphatases (Gee et al. 1999) and subsequently optimized for use in the high-throughput assay format (Welte et al. 2005). The DIFMUP-based assay may be helpful for the kinetic analysis and characterization of small-molecule

inhibitors for PTP, which have been implicated as negative regulators in insulin signalling (e. g. PTP α , LAR, CD45, TC-PTP, YOP, SHP2), in general, and PTP1B, in particular.

PROCEDURE

All reactions are carried out at 37°C in black polystyrol 96-well plates with flat bottoms. For measurement of PTP1B, PTP α , LAR, CD45, TC-PTP, YOP, and SHP2, the reaction buffer contains 50 mM HEPES (pH 6.9), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, and for VHR, the reaction buffer contains 50 mM sodium acetate (pH 5.6), 100 mM NaCl, 1 mM EDTA, 2 mM DTT. The substrate stock solution is prepared in DMSO at a final concentration of 10 mM and stored at -20°C. For measurement, DIFMUP is diluted 10-fold in the desired reaction buffer, yielding a final concentration of 1 mM. Fluorescence excitation of hydrolyzed DIFMUP (Molecular Probes) and the fluorescent standard DIFMU is measured at 358 nm and emission is detected at 455 nm in a fluorescence plate reader (e. g. Spectramax, Molecular Devices).

For kinetic analysis, the DIFMUP stock solution is pre-diluted in reaction buffer and added to the reaction mixture to yield final concentrations of 0–300 μ M. The assay is initiated by adding 10 μ l of substrate to 90 μ l of reaction buffer containing the prediluted PTP (final concentrations: PTP1B, 100 ng/ml; PTP α , 500 ng/ml; LAR, 125 ng/ml; CD45, 100 ng/ml; TC-PTP, 100 ng/ml; SHP2, 2 μ g/ml, YOP, 50 ng/ml).

Compounds/drug candidates are pre-diluted in reaction buffer containing 100 ng PTP1B to the indicated final concentrations ranging from 0.8 to 80 μ M in a final volume of 90 μ l. The reaction is started by adding 10 μ l of substrate buffer containing 1 mM DIFMUP and the intensity of released DIFMU is monitored continuously for 5 min. Malachite green and PNPP assays are performed as described (Zhang and Dixon 1994, Zhang et al. 1993). Initial velocities of the reactions are used to calculate kinetic constants using Sigma Plot software. The initial velocities are plotted against the inhibitor concentrations and the IC₅₀ is calculated.

Detailed analysis of the binding mode of compounds/candidate drugs is performed as follows. For analysis of the time dependence of inhibition, different inhibitors are incubated at their IC₅₀ concentrations with PTP1B for 0, 10, and 15 min. The reaction is started by adding 10 μ l of substrate buffer containing 1 mM DIFMUP and the reaction is followed for 10 min. For analysis of the reversibility of inhibition, compounds/drug candidates are incubated for 10 min

with PTP1B. After addition of DIFMUP to a final concentration of 10 μM , the reactions are followed continuously until the fluorescence emission do not increase further. The reactions are restarted by adding DIFMUP to a final concentration of 100 μM and the release of DIFMU is monitored for an additional 10 min.

EVALUATION

The fluorogenic substrate, DIFMUP, was developed as a substrate for phosphoserine/threonine phosphatases to enable continuous measurement of activity (Gee et al. 1999). Its hydrolysis by a phosphatase results in the release of fluorescent DIFMU, which can be easily followed in continuous mode by a fluorescence reader. A great advantage of this substrate is its low pK_a value of 4.7 compatible with pH 7.0 during the reaction without prior alkalization (Sun et al. 1998). Furthermore, the substrate yields a higher fluorescence quantum than other frequently used fluorogenic substrates, such as 4-methylumbelliferyl phosphate. This substrate is accepted a recombinant fragment of human PTP1B (Welte et al. 2003). This finding has meanwhile been extended by a detailed kinetic and comparative analysis of PTP reactions using DIFMUP and other substrates (Welte et al. 2005). Compared to PNPP, DIFMUP is a better substrate for PTP1B as reflected in both its lower K_m and higher turnover number. However, dephosphorylation of a phosphotyrosine-containing peptide derived from the IR is even more efficient, indicating the requirement of amino acids adjacent to the phosphoamino acids.

DIFMUP is accepted by other PTP from different families. These include CD45, PTP α , SHP2, YOP, LAR, and TC-PTP. For these PTP a clear relationship between the relative fluorescence signal and the enzyme concentration is detected, which, however, with regard to the protein amount required differed by two orders of magnitude between LAR, TC-PTP, CD45, YOP (50–500 μg) and PTP α , SHP2 (0.5–2 μg) with PTP1B and the closely related TC-PTP showing the highest affinity for DIFMUP (Welte et al. 2005). These findings hint at subtle differences in the catalytic sites between these PTP for hydrolysis of optimally bound DIFMUP. Taken together, differences in the substrate recognition and catalytic sites of the various PTP are presumably monitored in the course of DIFMUP binding and cleavage. In contrast, no major kinetic differences between a number of phosphatases were reported for the hydrolysis of 3,6-fluorescein diphosphate and other fluorogenic substrates (Huang et al. 1999, Wang et al. 1999), arguing for similar and more

flexible binding modes to the substrate recognition and catalytic sites of these apparently more unspecific substrates.

The search for inhibitors of PTPs requires a robust test system to assay a large number of compounds for a multitude of different enzymes. A novel non-peptidic inhibitor, a benzoxathiazoldioxide derivative (Petry et al. 2002, Liu et al. 2002) exhibits in different PTP1B assays using either DIFMUP, PNPP, or the IR phosphopeptide as substrate an IC_{50} of 3.8, 5.1 and 3.9 μM , respectively. DMSO used as solvent for the inhibitors did not affect cleavage of DIFMUP to up to 4% final concentration during the assay, reflecting the robustness of the assay.

PTP all share a nucleophilic cysteine residue located in the catalytic cleft, which is sensitive to oxidation or covalent modification (Zhang 2003, Johnson et al. 2002). Therefore, characterization of the nature of their inhibition by small-molecule inhibitors is important, which requires detailed kinetic studies. The possibility of continuous measurement of DIFMUP hydrolysis allows rapid characterization of the reversible nature of inhibition. For this, the enzyme is incubated in the absence or presence of inhibitor in the presence of 10 μM DIFMUP. After incubation for 10 min, when hydrolysis of DIFMUP stops due to substrate depletion (as revealed by approaching a plateau level of fluorescent DIFMU generated), a 10-fold excess of fresh DIFMUP is added to the reaction. This leads to complete reversion of inhibition of DIFMUP hydrolysis as reflected in the almost identical initial velocities of the reactions containing or lacking (control) the inhibitor after fresh DIFMUP has been added. The degree of inhibition is often found to depend strictly on the incubation time of the inhibitor with PTP1B prior to DIFMUP addition.

In summary, the high sensitivity and compatibility with a broad pH range of the fluorogenic DIFMUP assay provide advantages with regard to saving enzyme and miniaturization. Consequently, the DIFMUP assay will be useful for the development of throughput screening assays for a variety of physiologically important PTP as has been already been demonstrated for the receptor PTP, LAR (Pastula et al. 2003). In addition to its technical advantages, DIFMUP seems to reflect the binding mode of phosphotyrosine-containing peptide substrates more closely than the widely used non-peptidic substrates, PNPP and 3,6-fluorescein diphosphate. These characteristics may facilitate the future search for competitive and reversible PTP inhibitors as.

K.6.3.3.2**PTP Identification Using Substrate Trapping****PURPOSE AND RATIONALE**

To understand fully the role of PTP in cellular function, in general, and in insulin signal transduction, in particular, it will be necessary to identify multitude of the physiological substrates of the individual members of the PTP family. It has shown in numerous studies that alteration of the nucleophilic Cys to Ser or Ala allows some PTPs to be isolated in a complex with their target substrates. Thus it appears that the Cys \rightarrow Ser/Ala mutants may be utilized to isolate all combinations of PTP1B and its substrates. Additional alternative "substrate-trapping" PTP mutants have been identified by systematic mutational analysis of the invariant residues that were predicted from the crystal structure to be important in catalysis. In one such mutant form of PTP1B Asp-181, the residue that functions as a general acid to facilitate cleavage of the scissile P-O bond in the substrate, has been altered to Ala. Clearly, a definition of the spectrum of phosphotyrosyl proteins dephosphorylated by PTP1B *in vivo* will be a prerequisite for the elucidation of the physiological function of that enzyme. Such substrate identification or trapping may be achieved by the following method.

PROCEDURE**Immunoprecipitation, Immunofluorescence and Activity Assay**

Cells transformed or transfected with vectors coding for PP are lysed 44–48 h after transfection in 50 mM Tris/HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 5 mM iodoacetic acid, 10 mM sodium pyrophosphate, 10 mM NaF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM benzamidine (1 ml per 10-cm dish, 0.5 ml per 6-cm dish). PTP1B is immunoprecipitated from lysates with a monoclonal antibody (mAb). GST-PTP1B fusion proteins are precipitated with glutathione-Sepharose. Precipitates are collected by centrifugation (5,000 \times g, 15 s, 4°C), washed four times with 0.7 ml of ice-cold lysis buffer, and finally heated at 95°C for 5 min in 60 μ l of 2 \times Laemmli sample buffer. mAb to phosphotyrosine are typically used for immunoblotting at the following concentrations: 4G10 (Upstate Biotechnology) 2 μ g/ml, RC20b (Transduction Laboratories) 0.5 μ g/ml. The levels of expression of wild-type, C215A, and mutant PTP1B should always be equivalent. For immunofluorescence experiments, cells are seeded onto glass coverslips at $2.5\text{--}3 \times 10^5$ per 6-cm dish, transfected with 10 μ g of

PTP1B cDNA, wild-type or mutant, and processed for immunofluorescence staining at 36 h after transfection as described by Lorenzen and coworkers (1995).

EVALUATION

Considering that the substrate-trapping mutant, PTP have the ability, through interaction with phosphotyrosine residues, to increase the basal phosphorylation of their target substrates, one might expect them to promote ligand-independent signaling. However because these mutants form stable complexes with target substrates, they may also interfere with signaling *in vivo* in a manner analogous to the wild-type phosphatase. Thus, if the site of tyrosine phosphorylation on the substrate is critical for a protein-protein interaction required for signaling or if the substrate is an enzyme and the phosphorylation site is located close to the active site, steric hindrance resulting from binding of the mutant PTP may exert an effect that is functionally equivalent to dephosphorylation.

The successful application of this technique to identify additional substrates of PTP1B, and substrates of other PTP, will require expression of the Asp \rightarrow Ala mutant PTP in cells and selection of an appropriate stimulus to trigger tyrosine phosphorylation of the substrate and accumulation of a complex with the mutant PTP. For PTP1B, IR is naturally abundant in myocytes, hepatocytes and adipocytes, and its basal rate of autophosphorylation generates enough tyrosine-phosphorylated protein to be trapped by PTP1B-D181A and to be detected by anti-phosphotyrosine immunoblotting. Its high molecular weight, characteristic of growth factor receptors, facilitates identification. Of the less-phosphorylated substrates of PTP1B, the finding that phosphorylation of p70 is enhanced upon cotransfection with v-src and D181A may enable enough of this substrate to be isolated to allow its identification by peptide sequencing. In addition to the use of trapping mutants to identify novel PTP substrates, it also may be possible to identify the site within the cell at which a PTP acts on a particular target, through colocalization of the substrate and the Asp \rightarrow Ala mutant PTP.

These findings indicate that PTP1B can display substrate specificity *in vivo* and bolster the hypothesis that the subcellular targeting is an important mechanism by which such specificity is achieved. The substrate-trapping mutant introduced by Flint and coworkers (1997) is altered in a residue that is an invariant catalytic acid in all members of the PTP family. Therefore, the use of this mutation should be generally applicable to any PTP and may represent a powerful tool with which

to delineate the physiological substrate specificity of other members of the family, thereby revealing important insights into their function *in vivo* and their potential as drug targets.

K.6.3.3.3

Lipid Phosphatase Activity Measurement

PURPOSE AND RATIONALE

The methods currently available for the assay of lipid-metabolizing enzymes, in general, and lipid phosphatases, in particular, rely on the release of radioactive isotopes with some form of substrate/product separation step before quantitation. Estimation of cellular mass of phosphoinositides usually requires labeling of cells with [³H]inositol or [³²P]orthophosphate followed by deacylation and HPLC analysis. Estimation of phosphoinositide mass in tissues is at present restricted to PtdIns(3,4,5)P₃ and requires extraction of the lipids and removal of the phosphoinositol head groups followed by a radiometric displacement assay which also requires synthesis of radiolabeled inositol(1,3,4,5)tetrakisphosphate. These assays also suffer from the drawback that the labeled substrates or precursors required are expensive and samples require extensive processing before analysis.

A novel assay for quantification of phosphoinositides based on their ability to bind specifically to certain pleckstrin homology (PH) domains (Gray et al. 2003). PH domains are the major intracellular targets of PtdIns(3,4,5)P₃, PtdIns(3,4)P₂, and several other phosphoinositides. Recently the number of characterized PH domains binding inositol lipids with a broad range of affinity and specificity has increased (Lemmon and Fergusson, 2001). Other recently discovered phosphoinositide binding domains, like the phox homology domain (Xu et al. 2001, Bravo et al. 2001) and FYVE domains (Gillooly et al. 2001), could potentially also be used in the assay formats described herein. Other critical components of the assay concept are biologically active short acyl chain phosphoinositides that recently have become commercially available. Important structural features of these include short acyl side chains (diC4 to diC8), rendering them water soluble, and the addition of biotin to the terminus of the *sn*-1 acyl chain while still allowing selective recognition of the inositol head group. Finally, sensitive non-radioactive detection of lipid/PH domain complexes relies on either TR-FRET using Lance reagents (e. g. Perkin-Elmer Wallac) or the recently introduced AlphaScreen technology (BioSignal Packard). In both cases, PtdIns(3,4)P₂

or PtdIns(3,4,5)P₃ present in samples is detected in competition assays by its ability to dissociate signal-generating complexes between PH domain, biotinylated lipid, and detection reagents. The assay systems presented here allow the assay of lipid phosphatases in a homogeneous format compatible with throughput screening and, in the case of the TR-FRET format, capable of real-time kinetic measurements.

PROCEDURE

Detection of Biotinylated Phosphoinositide/PH Domain Complexes

The GRP1 PH domain (amino acids 263 to 380) is PCR cloned from a mouse brain cDNA library as described by Gray and coworkers (1999). The protein is expressed from the pGEX 4T1 vector (Amersham Pharmacia) in *Escherichia coli* and affinity purified on glutathione-agarose using the manufacturer's standard protocols. The purified PH domains are labeled with Lance chelate according to the manufacturer's protocols.

AlphaScreen detection is performed in 384-well microtiterplates in 50 mM HEPES/KOH (pH 7.4), 50 mM NaCl, and 0.1% BSA. Biotinylated, short-chain (diC6) phosphoinositides and GRP1 PH-GST are added at 15 and 3.75 nM, respectively. Donor and acceptor AlphaScreen beads (Perkin-Elmer) are added at 5 µg/ml to a final volume of 50 µl. Plates are incubated in the dark for 5 h to ensure binding is complete and then read in an AlphaQuest AD instrument (Perkin-Elmer) using standard settings. The TR-FRET sensor complex consists of 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM DTT, and 0.05% CHAPS with APC-streptavidin) 32 nM and 120 nM biotinylated, short-chain (diC6) phosphoinositides, and 35 nM Lance chelate labeled GST-PH domain. Alternatively the TR-FRET sensor complex contains 21 nM Lance chelate labeled anti-GST antibody and unlabeled PH domain in a final volume of 50 µl. All assays contain CHAPS at low concentration to prevent loss of lipids by adsorption onto plastic surfaces.

For all assays plates are read with the following settings: excitation 360–35 nm filter, emission 665 nm filter, dichroic filter 505 nm, PMT 1000 V set to digital sensitivity 2, 100 flashes per well, 10-ms interval between flashes, read 50 ms after flash, and integration 1000 ms.

The TR-FRET assays are conducted in 50 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 0.05% CHAPS (assay buffer) using a two-component reaction. The first component is a twofold-concentrated sensor complex consisting

of 32 nM APC–Streptavidin, 120 nM biotinylated diC6PtdIns(3,4,5)P₃, and 35 nM Lance chelate labeled GST–PH domain. Alternatively the sensor complex contains 21 nM Lance chelate labeled anti-GST antibody and unlabeled PH domain. The first component also contains 100 ng recombinant PI3-kinase γ in a final volume of 25 μ l assay buffer. The second component contains the diC8PtdIns(4,5)P₂ at two times the required final concentrations and ATP at 100 μ M again in 25 μ l assay buffer. The assays are started by mixing the two components in a 96-well plate and reading at the required time intervals to obtain rates of reaction.

EVALUATION

A novel approach to quantitation of phosphoinositides in cell extracts and *in vitro* enzyme-catalyzed reactions using suitably tagged and/or labeled PH domains as probes (Gray et al. 2003). Stable complexes are formed between the biotinylated target lipid and an appropriate PH domain, and phosphoinositides present in samples are detected by their ability to compete for binding to the PH domain. Complexes are detected using AlphaScreen technology or time-resolved FRET. The assay procedure has been validated using recombinant PI3-K γ with diC8PtdIns(4,5)P₂ as substrate and general receptor for phosphoinositides-1 (GRP1) PH domain as a PtdIns(3,4,5)P₃-specific probe. This PI3-K assay is robust and suitable for throughput screening. The approach is adaptable to lipid phosphatases as demonstrated by assays for PTEN, a phosphoinositide 3-phosphatase, which is measured using the same reagents but with diC8PtdIns(3,4,5)P₃ as substrate. PtdIns(3,4,5)P₃ present in lipid extracts of growth factor-stimulated Swiss 3T3 and HL60 cells is also detectable at picomole sensitivity. Gray and coworkers (2003) therefore proposed that similar procedures should be capable of measuring any known phosphoinositide present in cell and tissue extracts or produced in PK and PP assays by using one of several well-characterized protein domains with appropriate phosphoinositide-binding specificity

PtdIns(3,4,5)P₃ Measurements

Alternatively to the above method of quantification of phosphoinositides by using phosphoinositide-binding proteins, PtdIns(3,4,5)P₃ can be measured directly by prior radiolabeling as described by Blero and coworkers (2005). For this, cells (1.5×10^6), which have been pretreated with compounds/drug candidates, are cultured in 10% serum overnight. Cells are washed twice in medium without serum and twice in medium without either phosphate or serum. They are labeled

for at least 4 h in medium with [³²P]orthophosphate (250 μ Ci/ml) but without serum. Cells may be stimulated with compounds/drug candidates during the labeling period, in addition or alternatively. The reaction is terminated by 5 ml cold PBS. Cells are lysed in 3.75 ml of 2.4 N HCl. Lipids are extracted in 3 ml methanol and 4.5 ml CHCl₃. After TLC and deacylation of the phosphoinositides, separation is performed by HPLC on Whatman SAX columns (Pesesse et al. 2001). Radioactivity is estimated with an online detector (e.g. Raytest, Germany). PtdIns(4,5)P₂ is also determined by labelling the cells with [³H]inositol as reported by Serunian and coworkers (1991). The various 3-phosphorylated phosphoinositides standards have to be prepared in insulin-stimulated CHO-IR cells or in platelets as reported (Blero et al. 2001, Giuriato et al. 2003).

Measurement of Recombinant SHIP2 Activity

For the identification and characterization of compounds/drug candidates which directly interfere with the activity of SHIP2 and may represent future anti-diabetic drugs the activity of SHIP2 can be assayed with cells transfected with recombinant SHIP2 by HPLC analysis of their phosphoinositides after radiolabeling *in vivo* according to the procedure of Wada and coworkers (2001).

In Vivo Generation of ³²P-Labeled Phosphoinositides and HPLC Analysis

The same numbers of 3T3-L1 adipocytes transfected with LacZ, wildtype WT-SHIP2, or phosphatase-defective Δ IP-SHIP2 are phosphate-starved overnight in phosphate-free DMEM, followed by serum starvation for 3 h. [³²P]orthophosphate (0.1 mCi/ml) is then added, and the cells are cultured for an additional 2 h. Following the labeling period, the cells are incubated without or with compounds/drug candidates for 15 min. The reaction is terminated by washing once with ice-cold PBS, followed by the addition of methanol/1 N HCl (1/1). The labeling of the cells with [³²P]orthophosphate is conducted at the same time in all three sets of transfected cells. Phospholipids are then extracted with chloroform. The extracted lipid is deacylated and subjected to amino-exchange HPLC using a Partisphere strong anion-exchange column as described previously (Serunian et al. 1991, Funaki et al. 1999). The PI(3,4)P₂ and PI(3,4,5)P₃ levels in the same sample for each cell line are measured within a single HPLC run. The radioactivity is detected with an on-line radiochemical detector.

MODIFICATION OF THE METHOD

Lin and coworkers (2003) reported about the separation of phospholipids, such as phosphoinositides, in microfluidic chip devices and its application to homogeneous high-throughput screening assays for lipid-modifying enzymes. The method takes advantage of the high-separation power of the microchips that separate lipids based on micellar electrokinetic capillary chromatography (MEKC) and the high sensitivity of fluorescence detection. The assay format consists of two steps: an on-plate enzymatic reaction using fluorescently labeled substrates followed by an on-chip MEKC separation of the reaction products from the substrates. The utility of the assay format for screening is demonstrated using phospholipase A₂ but may be extended to other phospholipases and lipid phosphatases with the advantages of avoidance of the use of radioactive substrates and tedious separation/washing steps and the detection of both substrate and product simultaneously.

K.6.3.3.4

Generic Assay for Protein Kinases (PK) and Phosphatases (PP) Based on Phosphate Release

PURPOSE AND RATIONALE

PK and PP produce or consume inorganic phosphate (P_i). Detection of an increase or decrease of the P_i concentration in the environment of these enzymes is a common way to monitor the enzymatic activity. Assays capable of measuring a change in the inorganic phosphate concentration serve as uniform readout for the activity of these enzymes (Ellen Chan and Swaminathan 1986, Brune et al. 1994). A number of colorimetric and fluorometric assays have been reported for the determination of inorganic phosphate (Itaya and Ui 1966, Kirkbright et al. 1972, Sebbon and Fynn 1973). Most of these assays are based on complexation reactions with the free phosphate. In addition, some methods for phosphate measurements have been developed based on the enzymatic conversion of phosphate and a substrate to a UV-absorbing product (Webb 1992, Ungerer et al. 1993). The disadvantages of all these methods are that they are slow, lack sensitivity or are sensitive to interfering compounds (Ellen Chang and Swaminathan, 1986, Tashima 1975, Hwang 1976).

To circumvent these problems, Brune and coworkers (1994, 1998) developed a continuous-flow phosphate assay. As an improvement Schenk and coworkers (2003) introduced a probe for the rapid measurement of P_i, in particular to follow its release in real

time from enzymes such as phosphatases. The probe consists of phosphate-binding protein (PBP) labeled with a fluorophore, *N*-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide (MDCC). Upon binding of P_i under physiological conditions a ~13-fold increase in fluorescence is observed. The binding of P_i to MDCC-PBP is very fast, has a high affinity, and is very sensitive. These characteristics make the reaction between P_i and MDCC-PBP very suitable for dynamic P_i detection assays and thus for application in a continuous-flow phosphate assay. A further increase in sensitivity of a continuous-flow generic fluorescent assay for PK and PP was achieved by Schenk and coworkers (2003) by coupling to liquid chromatography (LC).

K.6.3.3.5

Cellular PTP Assays

The development of cell-based assays for the identification as well as evaluation of the biological activity of PTP inhibitors which positively affect insulin signaling and reduce insulin resistance (e. g. PTP1B, see above) is hampered by a number of issues. The typical functional readout for PTP1B inhibition has been to measure IR phosphorylation (see K.6.3.1.3). Yet, the increase in IR phosphorylation due to PTP1B inhibition is usually variable, dependent on cell type and at most two- to three-fold over controls, thus, making it unsuitable for screening. Furthermore, because many of the current potent PTP1B inhibitors contain a phosphonate or a phosphate mimetic they are highly charged and therefore cell permeability becomes an issue. Without a robust cellular functional readout and questionable cell permeability of PP inhibitors, it becomes difficult to reconcile the reason for the lack of activity of PTP inhibitors in cell-based assays. In order to circumvent some of these issues, yeast- and insect cell-based PTP1B assays have been developed.

K.6.3.3.5.1

Yeast-Based Assay

PURPOSE AND RATIONALE

Yeast has been used as a model system for the screening of various compounds/drug candidates. It provides a cellular environment to prevent PP oxidation and measuring yeast growth is both an easy and sensitive screening assay (Melese and Hieter 2002). In addition, yeast cell permeability has been shown not to be an issue in concentrating compounds internally, but like mammalian cells, the rate-limiting factor in internalization of compounds has been due to the function of

efflux pumps. v-Src, a tyrosine kinase from the Rous Sarcoma virus, is lethal when overexpressed in the yeast *Saccharomyces cerevisiae* (Brugge et al. 1987). The lethality of v-Src has been attributed to the hyperphosphorylation of multiple yeast proteins leading to mitotic dysfunction but the exact target responsible for the toxicity remains to be identified (Kornbluth et al. 1987, Florio et al. 1994, Trager et al. 1997). Yeast can be rescued from the v-Src lethality by co-expressing the catalytic domain of PTP1B (Florio et al. 1994). This rescue is presumably due to the activity of PTP1B in dephosphorylating the various yeast proteins phosphorylated by v-Src. Based on these observations Montalibet and Kennedy (2004) developed and optimized a yeast-based assay to screen for PTP1B inhibitors.

PROCEDURE

Yeast with leucine and uracil deficiency markers are transformed with the plasmids coding for v-Src and the appropriate PTP (here PTP1B) and the colonies formed three days after are transferred to leucine and uracil dropout media containing raffinose as the sole carbon source and grown overnight. Serial dilutions of the inhibitors are arrayed in 96-well plates containing leucine and uracil dropout media with 4% galactose. The plates are inoculated with the overnight yeast cultures at a final concentration of 10^6 per ml in the absence or presence of increasing concentrations of compounds/drug candidates (Hammonds et al. 1998). The wells are overlaid with mineral oil, incubated at 30°C and read periodically at 600 nm for 4 days. The growth curves generated are used to obtain EC_{50} -values for the putative PTP1B inhibitors.

EVALUATION

Based on the previous observation that co-expression of PTP1B can rescue yeast from v-Src lethality, Montalibet and Kennedy (2004) optimized the expression levels of PTP1B and v-Src such that inhibition of PTP1B activity results in growth interference. PTP1B catalytic activity was shown to be absolutely required for yeast growth since the catalytically inactive PTP1B mutants C215S and D181A failed to overcome the lethality of v-Src. A certain stoichiometry between PTP1B and v-Src expression is required to obtain a robust growth phenotype and at the same time to ensure sensitivity toward inhibitors. Screening can be carried out in 96-well plates and growth of the liquid culture measured by absorbance at 600 nm. The PTP inhibitor, vanadate, specifically inhibited PTP1B-dependent growth in a concentration-dependent man-

ner with an EC_{50} of 0.92 mM. This simple yeast growth interference assay may be useful for throughput screening for inhibitors of PTP1B, in particular, and PTP, in general.

K.6.3.3.5.2

Insect Cell-Based Assay

PURPOSE AND RATIONALE

Cromlish and coworkers (1999) described an intact cell-based assay for the discovery of cell-permeable, selective PTP inhibitors utilizing the baculovirus expression system, where a PTP of interest is expressed in Sf9 insect cells and a direct readout of the PP activity within these cells is obtained from the amount of hydrolysis of the cell-permeable substrate, *para*-nitrophenylpyrophosphate (pNPP).

PROCEDURE

Baculoviruses recombinant for the cDNA coding for the desired PTP (e. g. PTP1B) is prepared using the Bac-to-Bac Baculovirus Expression System and recovered from the supernatant medium from transfected Sf9 insect cells after three rounds of amplification to up to a total viral stock volume of 500 ml. Sf9 cells are infected with the recombinant or control baculovirus (Cromlish and Kennedy 1996), collected 29 hpi by centrifugation ($48 \times g$, 5 min), washed once in assay buffer (Hanks' solution buffered with 15 mM HEPES/KOH, pH 7.4) and re-centrifuged ($21 \times g$, 10 min). The cells are resuspended gently in assay buffer and examined using a hemocytometer and microscope for cell density as well as trypan blue exclusion. Assays are performed using a pipetting robot, programmed to mix gently after each addition. 200- μ l Hanks' solution containing the cells. Following a 15-min incubation with compounds/drug candidates at 37°C, the cells are challenged with 1–50 mM of tissue culture-grade pNPP for 15 min. The cells are centrifuged ($410 \times g$, 3 min, 4°C). 100- μ l samples of the supernatants are transferred to fresh clear polystyrene 96-well plates, and the amount of hydrolysis of pNPP is determined spectrophotometrically at OD of 405 nm. The pNPP hydrolysis window obtained between PTP-expressing cells and control-infected cells represents the amount of phosphatase activity due to the PTP of interest.

EVALUATION

Inhibitions are calculated by comparing pNPP hydrolysis of the inhibitor-treated PTP-expressing cells with that of DMSO-treated PTP-expressing cells. For the PTP Sf9 cell-based assay to be useful in screening for

PTP inhibitors, it is necessary to harvest the cells at a time point when sufficient amounts of the protein of interest are produced, yet the cells are healthy and viable prior to the deleterious effects associated with the later stages of the viral infection.

K.6.3.3.5.3

Bacterial Cell-Based Assay

PURPOSE AND RATIONALE

Senn and Wolosiuk (2005) developed a high-throughput screening assay for the detection of phosphatase activity in bacterial colonies overexpressing mammalian phosphatases. Unlike the insect cell-based method, this procedure is compatible with the use of physiological substrates but nevertheless is versatile, since it is based on the detection of the common product of all phosphatase reactions, P_i . In this method, substrates diffuse from a filter paper across a nitrocellulose membrane to bacterial colonies located on the opposite face, and then the reaction products flow back to the paper. Finally, a colorimetric reagent discloses the presence of orthophosphate in the filter paper.

PROCEDURE

Escherichia coli cells are transformed with vectors coding for the corresponding PP under a strong inducible promoter and after appropriate dilution grown directly over a nitrocellulose membrane that lay on Luria-Bertani (LB) agar supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin. When the diameter of colonies is 1 mm (~ 24 h), the expression of the PP is induced by spreading 0.2 ml of inducer (e. g. 40 mM IPTG, 10% agarose) onto the agar surface. After 3 h, the nitrocellulose membrane (pore size 0.2 μm ; Bio-Rad, USA) is withdrawn from the Petri dish and placed with bacteria facing up for 10 min on a Whatman filter paper soaked in water. After this washing step has been repeated twice, the nitrocellulose membrane is similarly placed for 5–10 min on another Whatman filter paper previously soaked in the corresponding solution for catalysis. The catalytic solution employed to detect the PP activity is 50 mM Tris/HCl (pH 7.8), 10 mM MgCl_2 , and 0.1–1 mM phosphopeptide designed according to the consensus sequence for the relevant PP. The nitrocellulose membrane is withdrawn and can be stored for additional PP assays. The filter paper is first dipped gently for 2 s into the reagent (1.2 N sulfuric acid, 0.5% ammonium molybdate, 2% ascorbic acid) of Chen and coworkers (1956) for the assay of P_i and subsequently placed onto an absorbent paper to remove the excess of liquid until blue spots appeared.

EVALUATION

The bacterial cell-based procedure is sensitive enough to detect endogenous phosphate activity of bacteria under some experimental conditions. Although this feature appears to be useful for searching bacterial phosphatases, it can be a drawback when studying cloned mammalian PP. In those cases, *E. coli* strains with diminished phosphatase activity at the pH of interest should be used. In addition, plasmids of a high number of copies could be employed to reduce the time of catalysis and the background signal generated by chromosomal phosphatases. In most cases, the differentiation between the cloned PP activity and background phosphatase activity should be feasible.

Many currently used high-throughput procedures for the detection of compounds modulating PP activity are based on non-physiological substrates holding a colored or fluorescent moiety that facilitates the analysis of the reaction (Wahler and Reymond 2001) but generally impairs the intrinsic activity (Goddard and Reymond 2004). The bacterial cell-based assay for phosphatase activity offers the advantages of the use of specific protein or peptide substrates and the possibility of testing different experimental conditions using the same bacterial lawn. This feature may be helpful for the evaluation of the selectivity of PP.

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K.6.3.4

Expression, Phosphorylation, Activity and Interaction of Insulin Signaling Components

PURPOSE AND RATIONALE

Signal transduction from the cell surface to the nucleus was found to occur predominantly by serine/threonine- and tyrosine-specific PK participating in cascades involving phosphorylation of multiple substrates. The role of protein phosphorylation in signal transduction

is well characterized in the case of the insulin signaling cascade in insulin-responsive target cells, such as liver, muscle and adipose cells (for reviews see Chen 2006, White et al. 1994, 1998, 2003).

In the past decade, it has been increasingly recognized that insulin resistance is associated with chronic, low-grade systemic inflammation. These cellular inflammatory responses are thought to be mediated by serine/threonine phosphorylation by PK, such as IKK β (Yuan et al. 2001, Gao et al. 2002), JNK1 (Hirosumi et al. 2002), PKC θ (Yu et al. 2002), or direct interaction with inhibitory proteins, such as SOCS (Mooney et al. 2001, Steppan et al. 2005, Ueki et al. 2004), of key components of insulin signaling, such as the IRS proteins, which thereby undergo inactivation or down-regulation. The molecular mechanism of insulin-like compounds/drug candidates can be studied in appropriate assay systems monitoring the phosphorylation state or protein-protein interaction of the individual insulin signaling components.

K.6.3.4.1

Preparation of Cytosolic Extracts

PROCEDURE

Cultured Adipocytes

Cultured mouse or human adipocytes are incubated with insulin/compounds/drugs in 12- or 24-well plates for 10–20 min, then washed twice with 50 mM HEPES/KOH (pH 7.5), 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM EDTA, 10 mM glycerol-3-phosphate, 10 mM Na₄P₂O₇, 2 mM Na₃VO₄, 100 mM NaF. Cells are solubilized in the above buffer containing 1% (by vol.) Triton X-100, 0.1% sodium deoxycholate, 10% glycerol and protease inhibitors (20 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 50 μ g/ml aprotinin, 10 μ M E-64, 0.5 mM PMSF = lysis buffer) by scraping with a Teflon policeman (adherent cells). Total lysates are centrifuged (25,000 \times g, 20 min, 18°C). The infranatant is aspirated taking care to avoid contamination by the upper fat layer and recentrifuged to obtain the defatted cell lysate (cytosolic extract).

Primary Rat Adipocytes

Adipocytes (1–3 \times 10⁶ cells/ml) are incubated with insulin/compounds/drug candidates and then separated from the medium by flotation and lysed in 1 ml of chilled buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na₃VO₄, 50 mM Na₄P₂O₇, 10 mM NaF, 10 mM glycerol-3-phosphate, 0.2 mM PMSF, 20 μ g/ml leupeptin, 10 μ g/ml pepstatin, 25 μ g/ml aprotinin by

10 strokes in a loose-fitting Teflon-in-glass homogenizer at 15°C. The fat-free homogenate, prepared by centrifugation (500 × g, 3 min, 15°C), is supplemented with Triton X-100 (1% final conc.) and sodium deoxycholate (0.5% final conc.), incubated for 30 min at 4°C and finally centrifuged (13,000 × g, 10 min).

Liver

Portions of frozen liver (0.2–1 g wet weight), which has been perfused as described (see K.5.2.1) are homogenized in a buffer containing 50 mM HEPES/KOH (pH 7.4), 140 mM NaCl, 250 mM sucrose, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM EDTA, 2.5 mM Na₃VO₄, 10 mM glycerol-3-phosphate, 20 mM NaPP₁, 20 mM NaF, 1 mM phenylphosphate, 5 μM okadaic acid (sodium salt), 1% Nonidet P-40, 10% glycerol and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstatin A, 75 μg/ml aprotinin, 100 μM benzamidine, 2 μg/ml antipain, 10 μg/ml soybean trypsin inhibitor, 5 μM microcystin, 5 μM E-64, 0.2 mM PMSF) using an Ultraturrax T25 basic (three 10-s cycles at 2,000 rpm on ice) and then a tight-fitting Teflon-in-glass homogenizer (5 strokes at 500 rpm on ice). The total homogenate is centrifuged (48,000 × g, 30 min, 4°C). The supernatant is carefully removed to avoid contamination with the upper fat layer and re-centrifuged. The fat-free supernatant obtained is stored in liquid N₂ and used as cytosolic extract (3–5 mg protein per ml).

Diaphragm

After incubation with insulin/compounds/drug candidates, the hemidiaphragms (80–100 mg wet weight) are rapidly liberated from the rib cage, rinsed once with homogenization buffer (25 mM Hepes/KOH, pH 7.4, 140 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM sodium vanadate, 50 mM sodium pyrophosphate, 100 mM NaF, 10 mM glycerol-3-phosphate, 0.2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 μg/ml antipain, 25 μg/ml aprotinin), frozen in liquid N₂ and then homogenized in 2 ml of ice-cold homogenization buffer in a porcelain mortar on ice. After centrifugation (1,500 × g, 10 min, 4°C), the fat-free supernatant is supplemented with Triton X-100 (0.5% final conc.), incubated (30 min, 4°C) and centrifuged (18,000 × g, 20 min, 4°C) to yield the cytosolic extract.

Skeletal Muscle

Rats at week 10 of age and housed in a temperature-controlled room (22°C) with a 12:12-h light-dark cycle with free access to chow and water are deeply anesthetized with an i.p. injection of pentobarbital sodium (50 mg/kg). Intact epitrochlearis mus-

cles (type II muscle consisting primarily of both type IIa and IIb fibers) and strips of soleus muscles (consisting primarily of type I fibers) are prepared (~30 mg). Soleus strips and epitrochlearis muscles are incubated for 60 min at 37°C in 3 ml of oxygenated (95% O₂/5% CO₂) KHB containing 14 mM NaHCO₃ and then for 10 min with KHB supplemented with 8 mM glucose, 32 mM mannitol, 0.1% BSA in the presence of insulin/compounds/drugs. Thereafter, the muscles are rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, then trimmed of fat and connective tissue, quickly frozen between aluminium blocks cooled with liquid N₂ and weighed. Frozen muscles are homogenized in 8 vol. of ice-cold lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM glycerolphosphate, 10 mM NaF, 2 mM Na₃VO₄, 2 mM EDTA, 1% TX-100, 10% glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, 2 mM PMSF). Lysates are incubated on ice for 20 min and then centrifuged (13,000 × g, 20 min, 4°C) to yield the cytosolic extract.

K.6.3.4.2

Immunoprecipitation

Up to 1 -ml portions of cytosol (equal amounts of protein) are precleared (30 -min incubation at 4°C, 2-min centrifugation at 10,000 × g) with protein A/G-Sepharose (50 mg, Pharmacia) and then supplemented with typically 2–10 μg/ml appropriate antibodies: anti-IRS-1 (rabbit, polyclonal, protein A-purified, raised against a peptide corresponding to amino acids 1220–1235 of rat IRS-1, Upstate Biotechnology), anti-IRS-2 (raised against peptide mixture corresponding to amino acids 618–747 and 976–1094 of mouse IRS-2), anti-IRβ (raised against a peptide corresponding to the 100 carboxy-terminal amino acids of human IR, Upstate Biotechnology), anti-GSK-3β (mouse monoclonal, protein G-purified, raised against a peptide corresponding to amino acids 203–219 of *Drosophila* GSK-3β, Upstate Biotechnology, preadsorbed on protein A-Sepharose). After incubation (4 -20 h, 4°C, end-over-end rotation) and centrifugation (10,000 × g, 2 min, 4°C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM HEPES/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 20 mM glycerol-3-phosphate, 10 mM NaP₁, 2.5 mM Na₃VO₄) containing 1% Nonidet P-40 (omitted for sequential immunoprecipitation), then twice with 1 ml each of im-

munoprecipitation buffer containing 150 mM NaCl and 0.1% NP-40 and once with 1 ml of immunoprecipitation buffer containing 150 mM NaCl and twice with immunoprecipitation buffer lacking detergent and salt. The washed immune complexes are finally suspended in 50 μ l of Laemmli buffer (2% SDS, 5% 2-mercaptoethanol), heated (95°C, 2 min) and centrifuged. The supernatant samples are analyzed by SDS-PAGE. 4–12% Bis-Tris precast gel, pH 6.4, morpholinoethanesulfonic acid (MES)/SDS running buffer, e. g. Novex) under reducing conditions. For sequential immunoprecipitation, the supernatant samples (50 μ l) are supplemented with 1 ml of immunoprecipitation buffer containing 1% NP-40 and 1–10 μ l of the relevant antiserum. After incubation (12 h, 4°C), 50 μ l of protein A-Sepharose (100 mg/ml in immunoprecipitation buffer) is added and the incubation continued (4 h, end-over-end rotation). The immunocomplexes are collected, washed and processed for SDS-PAGE.

K.6.3.4.3

Immunoblotting

Immunoblotting is performed as described previously by Towbin (1979), Frick and coworkers (1998) and Müller and coworkers (2000) with minor modifications. Briefly, after SDS-PAGE and electrophoretic transfer of the proteins (2 h, 400 mA in 20% methanol, 192 mM glycine, 25 mM Tris, 0.005% SDS) to PVDF membranes (Immobilon, Millipore, Germany), the blocked membrane (1 h in blotting buffer containing 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 0.1% Brij, 0.01% NP-40 and supplemented with either 1% ovalbumin or 1% BSA or 5% non-fat dried milk according to manufacturer's instructions and the unspecific background signal obtained) is incubated (2 h, 25°C) with the appropriate antibodies (often 1:200–1:5,000). The membranes are washed (four times with Tris-buffered saline (TBS) containing 1% (by vol.) Nonidet-P40 and 0.5% Tween 20, twice with TBS containing 0.5% Tween 20, three times with TBS containing 0.05% Tween 20, twice with TBS). After incubation (1 h, 25°C) of the membranes with either [¹²⁵I]protein A (5 μ Ci/ml, Amersham-Pharmacia) or secondary goat anti-mouse or anti-rabbit antibody conjugated with horse-radish peroxidase (HRP, Santa Cruz Biotechnology) in the same blocking medium, the membranes are washed five times and then subjected in case of the radioactive method to autoradiography (Kodak X-Omat AR) or phosphorimaging (Molecular Dynamics, Storm 860) or in case of the

non-radioactive method to an enhanced chemiluminescence (ECL, Amersham-Pharmacia or Renaissance Chemiluminescence Detection System, NEN/DuPont) detection system.

EVALUATION

The band intensities on the autoradiographs are quantified on a scanning densitometer. The ECL-blot images are visualized by a LumiImager (Roche) and quantified using IMAGEQUANT software (Roche). The recovery in the amounts of immunoprecipitated protein is corrected (data on fold- or % stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting. Each experiment should be performed with samples from at least four different cell incubations or tissue preparations with antibody incubations/blots performed in triplicate, each.

K.6.3.4.4

Immunocomplex Kinase Assay

PURPOSE AND RATIONALE

In case of high expression and/or high specific activity, the relevant PK can be assayed directly using total cytosolic extracts from cells/tissues which have been incubated with compounds/drug candidates, provided the assay used displays sufficient specificity (e. g. due to specific substrate peptide). However, in most cases the relevant kinase has to be enriched by specific immunoprecipitation (see K.6.3.4.2) using antibodies which do not interfere with the subsequent measurement of the PK activity within the immunocomplex. This immunocomplex kinase assay also eliminates the need for specific peptide or protein substrates not accepted by PK which may be contained in the total cytosolic extracts used and lead to unspecific activity measurements.

The immunocomplexes prepared as described (see K.6.2.9.2) are washed in kinase buffer (50 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 5 mM MnCl₂, 1 mM MgCl₂, 0.5 mM DTT, 20 mM NaF, 1 mM Na₃VO₄) and then suspended in 25 μ l of kinase buffer. The PK reactions are started by addition of ATP (unlabeled or ³²P- or ³³P-labeled, final conc. 5 μ M, 0.2 mCi/ml to up to 100 μ M, 0.5 mCi/ml) and incubated (often 3–15 min, 22°C) in the absence (autophosphorylation) or presence of recombinant substrate protein (often 0.1–1 μ g) or peptide (5–25 μ g) in a final volume of 50 μ l. Autophosphorylation reactions are terminated by addition of 50 μ l of ice-cold 2-fold stop buffer (100 mM HEPES/KOH, pH 7.4, 300 mM NaCl, 200 mM ATP, 0.1% Triton X-100) and wash-

ing of the beads ($10,000 \times g$, 2 min, 4°C) twice with 1 ml of stop buffer and once with 1 ml of stop buffer lacking detergent and salt prior to addition of $20 \mu\text{l}$ of Laemmli sample buffer and boiling (95°C , 5 min). Substrate phosphorylation reactions are terminated by addition of $20 \mu\text{l}$ of 4-fold concentrated Laemmli sample buffer and boiling. The phosphoproteins contained in the supernatant of a spin ($10,000 \times g$, 5 min) are separated on SDS-PAGE (10% Bis-Tris resolving gel, morpholinopropanesulfonic acid/SDS running buffer) and analyzed by phosphorimaging directly (use of [$^{32/33}\text{P}$]ATP) or after immunoblotting with anti-phosphotyrosine or anti-pan-phosphoserine/threonine antibodies (use of unlabeled ATP). Under these conditions the PK reactions are linear with time for the assay period. Protein concentration is determined by the BCA protein assay with crystalline BSA as standard. Phosphorimaging is performed with a phosphorimager Storm 860 and quantitatively evaluated using ImageQuant software (Molecular Dynamics).

EVALUATION

Differences in recovery in the amounts of immunoprecipitated protein during a specific experiment are corrected in each case (data on fold- or % stimulation) for the amount of protein actually applied onto the gel by homologous immunoblotting. The data should be confirmed by running independent experiments with different batches of cytosolic fractions prepared from independent cell/tissue incubations, each, with two to five parallel independent immunoprecipitation/kinase assay analyses.

K.6.3.4.4.1

Phosphatidylinositol-3'-Kinase (PI3-K)

Portions of the anti-IRS-1/2 immunocomplexes (see K.6.3.4.4) are assayed for PI3-K activity by incubating in $100 \mu\text{l}$ of 20 mM Tris/HCl (pH 7.0), $50 \mu\text{M}$ [γ - ^{32}P]ATP ($5 \mu\text{Ci}$), 10 mM MgCl_2 , 2 mM MnCl_2 , 100 mM NaCl, 2 mM EDTA, $0.5 \mu\text{M}$ wortmannin (for control incubations, only) containing $10 \mu\text{g}$ of phosphatidylinositol and $1 \mu\text{g}$ of phosphatidylserine (Avanti Polar Lipids) for 15 min at 22°C . After addition of $20 \mu\text{l}$ of 8 M HCl and $160 \mu\text{l}$ of a 1/1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates (Silica Gel 60, Merck, Darmstadt) coated with 1% oxalate and developed in chloroform/methanol/water/ammonia (60/47/11.3/3.2, by vol.). Radiolabeled phosphatidylinositol-3-phosphate (PI3-P, average R_f value of 0.41 under these conditions) is visualized by autoradiography and quantitated by phosphorimage analysis. For

calculation of wortmannin-sensitive PI3-K, all values are corrected for PI3-P radiolabeled in the presence of wortmannin.

K.6.3.4.4.2

Phosphoinositide-Dependent Kinase-1/2 (PDK-1/2)

Akt-1/2 represents the major substrate of PDK-1/2 within the insulin signaling cascade (see K.6.3). Its phosphorylation can therefore be used as measure for specific increases in PDK-1/2 activity in response to insulin/compounds/drug candidates. For this, cytosolic extracts are prepared (see K.6.3.4.1) from cells/tissues which have been incubated with insulin/compounds/drug candidates. After centrifugation ($150,000 \times g$, 60 min, 4°C), 1-ml portions of the supernatant are immunoprecipitated with $10 \mu\text{g}$ anti-Akt-1/2 antibodies coupled to protein G-Sepharose beads. The immunoprecipitates are washed and finally suspended in $50 \mu\text{l}$ of Laemmli sample buffer (2% SDS), heated (95°C , 2 min) and centrifuged ($12,000 \times g$, 2 min). After dilution with 1 ml of immunoprecipitation buffer containing $10 \mu\text{g/ml}$ aprotinin, $20 \mu\text{g/ml}$ pepstatin, $10 \mu\text{g/ml}$ leupeptin, 1 mM PMSF and subsequent centrifugation again ($12,000 \times g$, 2 min), the supernatant is supplemented with $20 \mu\text{g}$ of a kit containing four different monoclonal anti-phosphoserine antibodies (Biomol, Hamburg, Germany; clones 1C8, 4A3, 4A9, 4H4) and the monoclonal anti-phosphothreonine antibodies (clone 1E11). After incubation (4 h, 4°C), the antibodies complexes are precipitates during a 2-h incubation with $50 \mu\text{l}$ of 50 mg protein A-Sepharose per ml of 50 mM HEPES/KOH (pH 7.4), 0.1% Triton X-100. The collected immunoprecipitates ($12,000 \times g$, 2 min) are washed three times with 1 ml each of 50 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 100 mM NaF, 0.2 mM Na_3VO_4 , 0.1% SDS, 1% Triton X-100 and finally twice with buffer lacking Triton X-100. Phosphoproteins are eluted from the washed precipitates by incubation with $500 \mu\text{l}$ of 50 mM HEPES/KOH (pH 7.4) containing 50 mM *p*-nitrophenylphosphate for 30 min at 4°C . The supernatants obtained by centrifugation are precipitated with TCA. The acetone-washed pellets are suspended in $50 \mu\text{l}$ of Laemmli sample buffer and analyzed by SDS-PAGE. Phosphorylated Akt-1/2 is detected by immunoblotting with anti-Akt-1/2 antibodies as described above. In case of high expression/phosphorylation of Akt, immunoblotting with the anti-AktpS473 (PDK-1) or pT307 (PDK-2) antibody (1:400) may be sufficient for the direct detection of Akt phosphorylation and consequently for evaluation of PDK-1/2 activity.

K.6.3.4.4.3**Protein Kinase B (Akt-1/2)**

Cytosolic fractions prepared from cells/tissues which have been incubated with insulin/compounds/drug candidates. 900 μ l of cytosolic extract are incubated with 100 μ l of anti-Akt-1/2 antibody (raised in rabbits by immunization with a synthetic peptide corresponding to residues 465–480 of the human PKB sequence, 10 μ g pre-coupled to 10 mg of protein A-Sepharose in 100 μ l of lysis buffer for 2 h at 4°C). The immunoprecipitates are collected by centrifugation (12,000 \times g, 2 min, 4°C), washed twice with 1 ml each of Akt assay buffer (20 mM Mops, pH 7.0, 1 mM EDTA, 1 mM EGTA, 10 mM Na₄P₂O₇, 50 mM NaF, 1 mM Na₃VO₄, 1 μ M microcystin, 1 μ M okadaic acid, 10 μ g/ml pepstatin, 25 μ g/ml leupeptin, 1 μ g/ml antipain, 10 μ g/ml aprotinin, 0.1 mM PMSF, 0.01% Brij35, 5% glycerol) containing 1% Nonidet P-40, 0.5 M NaCl and three times with 1 ml each of assay buffer lacking salt and detergent. The immunoprecipitates are finally resuspended in 45 μ l of PKB assay buffer containing 10 mM MgCl₂, 1 mM DTT, 2.5 μ M PKA inhibitor peptide (IP₂₀) and either the synthetic substrate peptide, Crosstide (100 μ M final conc.), which is based on the sequence surrounding the serine phosphorylation site of GSK-3 (Ser21 of GSK-3 α and Ser9 of GSK-3 β) or alternatively, histone 2B (150 μ g/ml). The assay is initiated by the addition of 5 μ l of [γ -³²P]ATP (50 μ M, 4 μ Ci) and terminated after incubation for 15 min at 30°C by placing the test tubes on ice. For determination of Crosstide phosphorylation, the 10- μ l portions of the samples are adsorbed on p81 phosphocellulose paper and extensively washed as described for the MAPK assay (see K.6.3.4.4.6). The radioactivity associated with the paper is counted by liquid scintillation counting. For determination of histone 2B phosphorylation, the samples are separated on a 12% SDS-PAGE. The autoradiogram is quantitated by densitometry. Exposure times are chosen which guarantee that the intensity of the bands is linearly related to the quantity of protein contained in the bands.

K.6.3.4.4.4**Glycogen Synthase-3 β (GSK-3 β)**

GSK-3 β activity is determined using immunocomplexes from cytosolic extracts of treated cells/tissues (see K.6.3.4.1 and K.6.3.4.2) with phospho-glycogen synthase peptide 2 (P-GS 2) as a substrate (Eldar-Finkelman et al. 1996, Wang et al. 1994). The GSK-3 β immunocomplexes (with antibodies raised in sheep by immunization with a synthetic peptide corresponding

to amino acids 471–483 of rat GSK-3 β and coupled to BSA) are precipitated with 2 mg of anti-sheep IgG coupled to agarose in 50 μ l of lysis buffer (under rotation for 2 h at 4°C). The beads are collected by centrifugation (12,000 \times g, 2 min), washed once with 1 ml of 100 mM Tris/HCl (pH 7.4) containing 0.2% Nonidet P-40, 0.5 M NaCl, 0.5 M LiCl, once with 10 mM Tris/HCl and twice with GSK-3 assay buffer (20 mM HEPES/KOH, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 10 mM NaF, 10 mM Na₄P₂O₇) and then suspended in 20 μ l of assay buffer containing 0.4 mg/ml BSA, 30 μ M [γ -³²P]ATP (3,000 Ci/mmol, 1 mCi/ml) and 20 μ M P-GS2. After incubation (15 min, 30°C), the reactions are terminated by addition of 20 μ l of 20% TCA and centrifugation (10,000 \times g, 5 min). Then, 15- μ l portions of the supernatant are spotted on 1 \times 1-cm pieces of Whatman P81 phosphocellulose paper. 20 s later, the filters are washed five times with 0.75% phosphoric acid (for at least 5 min each time) and once with acetone. The dried filters are counted for radioactivity in the presence of 5 ml of scintillation fluid (e.g. ACS, Amersham-Pharmacia). ³²P_i incorporation into the negative control peptide (glycogen synthase peptide 2 [Ala21]) is subtracted from values obtained using P-GS 2. No activity is typically measured with immunoprecipitates using non-immune IgG. In some experiments recombinant PP2A inhibitor (PP2AI, 1 μ g, Calbiochem) is used as substrate instead of P-GS1. In this case the PK reaction (conditions as above) is terminated by rapid centrifugation (12,000 \times g, 1 min, 4°C) and separation of the supernatant from the immunocomplex pellet. The supernatant of a precipitation (10% TCA for 1 h on ice, 15,000 \times g, 15 min, 4°C) containing the phosphorylated PP2AI is suspended in 50 μ l of 2-fold Laemmli sample buffer, heated (95°C, 5 min) and centrifuged (12,000 \times g, 2 min). The supernatants are run on SDS-PAGE and phosphorylated PP2AI and GSK-3 are visualized by autoradiography and quantitated by phosphorimaging.

K.6.3.4.4.5**MAPK/ERK Kinase (MEK)**

MEK activity is determined using immunocomplexes from cytosolic extracts of treated cells/tissues (see K.6.3.4.1 and K.6.3.4.2) with recombinant MAPK/ERK2 (Upstate Biotechnology) as substrate. The assay mix contains 4 mM MOPS buffer (pH 7.5), 5 mM glycerol-3-phosphate, 1.25 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM DTT, 10 mM MgCl₂, 0.15 mM ATP, and 0.04 μ Ci/ μ l [γ -³²P]ATP. The concentration of MAPK/ERK2 (0.5 ng/ μ l) is chosen to

approximate the concentration of MAPK/ERK2 that would be assayed in rat adipocyte cell lysates.

K.6.3.4.4.6

Mitogen-Activated Protein Kinase (MAPK)

MAPK activity is determined using immunocomplexes from cytosolic extracts of cells/tissues treated with compounds/drug candidates (see K.6.3.4.1 and K.6.3.4.2) with myelin basic protein (MBP) as substrate. Adipocytes incubated with insulin/compounds/drugs are washed with KRP-HEPES by flotation and then lysed in 1 ml of 50 mM HEPES/KOH (pH 7.2), 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5 mM Na₃VO₄, 40 mM *p*-nitrophenylphosphate, 10 mM glycerol-3-phosphate, 10 mM NaF, 0.2 mM PMSF, 25 µg/ml leupeptin, 25 µg/ml aprotinin by incubation for 30 min on ice and vigorous vortexing three times for 10 s at 10-min intervals. Lysates are cleared by centrifugation (12,000 × *g*, 10 min, 4°C). The infranatant below the fat cake is removed using a syringe, re-centrifuged and used for measurement of MAPK by addition of 250-µl portions to 250 µl of protein A-Sepharose (50 mg/ml of 50 mM HEPES/KOH, pH 7.2, 100 mM NaCl), which had been precoupled with 10 µl of rabbit anti-p42MAPK antiserum (raised against rat p42MAPK, Upstate Biotechnology) and 5 µl of rabbit anti-p44MAPK antiserum (raised against the carboxyl-terminal 14 amino acids of rat p44MAPK), and incubation for 2 h at 4°C. The beads are collected by centrifugation (12,000 × *g*, 2 min, 4°C), washed three times with 1 ml each of lysis buffer and three times with 1 ml each of 10 mM HEPES/KOH (pH 7.4), 10 mM MgCl₂ and then resuspended in 45 µl of kinase buffer (50 mM glycerol-3-phosphate, pH 7.3, 5 mM Na₄P₂O₇, 10 mM NaF, 0.5 mM EDTA, 15 mM MgCl₂, 2 mM DTT, 4.4 mM PK inhibitor peptide) containing 0.5 mg/ml MBP. The kinase reaction is initiated by the addition of 5 µl of 0.5 mM [γ -³²P]ATP (700 mCi/mmol). Following a 10-min incubation at 30°C, reactions are terminated by spotting 10 µl of the reaction mixture onto p81 phosphocellulose papers (Whatman), which are immediately immersed in 0.85% orthophosphoric acid under stirring and washed once in 95% ethanol for 5 min. Papers are dried and ³²P is quantitated by liquid scintillation counting.

K.6.3.4.5

Phosphoproteomics

GENERAL CONSIDERATIONS

The above methods for the detection of the phosphorylation state of insulin signalling components en-

compassing the IR and its downstream elements enable their individual analysis, only. Taking into account the expenditure in time and costs, their simultaneous analysis in a phosphoproteomic approach may be of great advantage. Innovations in the area of 2-D gel electrophoresis, protein analysis and computer databases are moving proteomics from futuristic possibilities into common laboratory procedures. The main aim of classic proteome studies is the separation of complex protein mixtures in order to visualize the relative levels of as many proteins as possible. Improvements of analytical techniques for protein identification, such as peptide mass fingerprinting, have enabled analysis of changes in proteomes on a large scale. However, obtaining information on co- and post-translational modifications within the analyzed proteome remains a major challenge. A wide variety of post-translational modifications, such as phosphorylation, glycosylation, acylation, methylation and acetylation, are known to play key roles in regulating the function, localization, binding specificity and stability of target proteins. To analyze this additional layer of protein diversity and to reveal its complexity, the traditional protein-by-protein approach clearly will not suffice to meet the huge magnitude of variation created by post-translational modifications. Therefore, development of proteome-based technology is required to analyze post-translational modifications of proteins, in general and phosphorylation, in particular.

PURPOSE AND RATIONALE

Since protein phosphorylation is an essential post-translational process in a variety of cellular processes, it is not surprising that perturbations in the equilibrium of PK and PP activities are fundamentally involved in metabolic diseases, such as type II diabetes and obesity. These pathogenetic changes in protein phosphorylation are preferably analyzed in parallel (“phosphoproteomics”) rather than sequential fashion (e. g. immunoblotting, immunoprecipitation).

PROCEDURE

Various techniques can be used to detect phosphorylated proteins. In many approaches detection of phosphorylated proteins separated on 2-D gels is activated by incubating cells with [³²P] or [³³P]orthophosphate. However, when radiolabeling is used to detect phosphoproteins it has to be considered that constitutively phosphorylated proteins with slow phosphate turnover rates, which hence only incorporate small amounts of radioactive phosphate, may be poorly detected. Furthermore, care is necessary when comparing ra-

tios of phosphorylated threonine, serine and tyrosine residues. Similarly, different metabolic phosphorylation/dephosphorylation rates cause unequal incorporation of [^{32}P] into different types of amino acids. The use of poly- and monoclonal antibodies directed against phosphoamino acids represents an alternative procedure to detect phosphorylated proteins blotted onto a membrane. This method is very sensitive since antibodies can detect as little as a few fmol of epitope. Among other strategies, antibodies recognizing phosphotyrosine (pY), phosphothreonine (pT) and phosphoserine (pS) have been generated by cross-linking of a phosphoamino acid-containing hapten to keyhole limpet hemocyanin or BSA. Anti-pT, pY and pS antibodies are commercially available and particularly pY antibodies are now widely used. It was found that different anti-pY antibodies bind to essentially the same proteins in immunoblotting analysis, independent of the exact nature of the immunogen against which the antibodies were raised. Little to no cross-reactivity to non-phosphorylated tyrosine, pT, pS, AMP or ATP has been observed. The sensitivity of the anti-pY antibody is very high, since tyrosine phosphorylation in normal and unstimulated cells and tissues is 100- to 1,000-fold less abundant than pS, accounting for only 0.02% of phosphoamino acids. However, phosphorylation-specific antibodies may not detect certain phosphorylated proteins due to steric hindrance of the recognition site, which is especially true for anti-pT and pS antibodies. In addition, lack of antigenicity conferred by pT and pS (in contrast to pY) has precluded rigorous and unquestionable detection of threonine and serine phosphorylation in proteins. More often pT and pS can be identified specially in the context of a larger epitope, only, thus precluding whole proteome analysis. However, many groups of substrates have overlapping epitopes, for example those with a proline in the +1 position of a phosphorylated Ser or Thr (the archetypal MAPK or CIK sites) for which monoclonal antibodies have recently been introduced. Once more common phosphorylation motifs have been determined, more antibodies will become available, thus facilitating phosphorylation analysis of the whole proteome (= phosphoproteomics; for a review see Kaufmann et al. 2001).

Extensive reviews cover the most critical tasks of lysis of mammalian cells, sequential extraction (Mollow et al. 1999), pre-fractionation (Cordwell et al. 2000), sample preparation (Herbert 1999, O'Farrell 1975), sample solubilization for isoelectric focusing (Rabilloud et al. 1997) and two-dimensional gel electrophoresis (Görg et al. 2000) and finally detection of

phosphoproteins by immunoblotting (see K.6.3.1.3.2.1 and K.6.3.4.3) and of total proteins by chemical staining (Dunn 1999, Birkelund et al. 1997)

EVALUATION

Detection of phosphorylated proteins within complex protein mixtures separated by 2-D gel electrophoresis followed by immunoblotting represents a relatively simple and sensitive method. Antibodies directed against pT, pS and pY have been successfully used in proteome studies to identify and characterize phosphoproteins. A strategy is recommended that allows high-throughput identification of phosphorylated proteins. The protein-by-protein approach for elucidating complex biological regulatory processes mediated by protein phosphorylation can now be replaced by analysis of the phospho-proteome under varying conditions.

However, the described methodology has its limits. Especially specific anti-pS and anti-pT antibodies are not able to recognize all proteins harboring phosphates on these residues. Therefore, immunoblotting analysis should be combined with radiolabelling techniques to obtain highly accurate and complete pictures of the phospho-proteome. A major challenge in creating phospho-proteome maps remains the low abundance of many phosphorylated key regulatory proteins, such as signal transduction molecules. Improvement of the sensitivity of protein identification techniques, such as peptide-mass fingerprinting by matrix-assisted laser-desorption-ionization-time-of-flight (MALDI-TOF) mass spectrometry will be the key to overcome this obstacle.

K.6.3.4.6

Multiplex Bead Immunoassay

PURPOSE AND RATIONALE

The introduction of methodologies that allow the simultaneous measurement of interrelated markers, e. g. phosphoproteins within a common signal transduction pathway, has revolutionized the drug discovery and evaluation process. Monitoring multiple proteins in a single sample and experiment saves time, labor costs and sample volume. The multiplex bead immunoassay developed by BioSource Inc. (CA, USA) in conjunction with the Luminex xMAPTM detection system and fluorescently encoded microspheres enables the simultaneous measurement of up to 100 different biological markers depending on the availability of appropriate (phosphosite-specific) antibodies. Each microsphere is labeled with a distinguishable fluorophore that allows

it to be assigned or gated to a particular region by the scanner. Antibodies specific for the (phospho-)protein of interest, are covalently linked to beads of a unique fluorescent region. The combination of different beads allows the user to simultaneously measure various protein markers of interest. The reagent and antibody bead kits offered by BioSource are intended for the quantitative determination of the (phospho-)protein in total cellular lysates and tissue homogenates.

The multiplex bead immunoassay is a solid phase sandwich immunoassay, which is designed to be analyzed with a Luminex 100™ instrument. The spectral properties of 100 distinct bead regions can be monitored with this instrument, a capability that affords this assay system the potential for measuring up to 100 different marker (phospho-)proteins in a single sample. Beads of defined spectral properties conjugated to marker-specific capture antibodies and samples (including standards of known marker concentration, blank controls etc.) are pipetted into wells of a filter bottom microplate and incubated for 2 h. During this first incubation, the marker (phospho-) proteins bind to the capture antibodies on the beads. After washing the beads, marker (phosphosite-) specific detector antibodies are added and incubated with the beads for 1 h. During this second incubation, the marker specific detector antibodies recognize their (phosphorylated) epitopes and bind to the appropriate immobilized marker (phospho-)proteins. After removal of excess detector antibodies, an anti-rabbit R-phycoerythrin (RPE) conjugated secondary antibody is added for 30 min. During this final incubation, the anti-rabbit RPE binds to the detector antibodies associated with the immunocomplexes on the beads, forming four-membered solid phase sandwiches. After washing to remove unbound anti-rabbit RPE, the beads are analyzed with the Luminex 100™ instrument. By monitoring the spectral properties of the beads and the amount of associated RPE fluorescence, the concentration of one or more analytes can be determined.

The multiple bead immunoassay has been initially introduced for the determination of the concentration of a panel of cytokines circulating in human serum (e. g. of patients suffering from primary Sjorens syndrome or bacterial infection; Szodoray et al. 2004, Pickering et al. 2004), but can meanwhile be applied successfully for the analysis of phosphorylation state of a number (currently seven) of key components of the insulin signal transduction cascade undergoing either tyrosine phosphorylation (IR, IGF-1 receptor, IRS-1) or serine/threonine phosphorylation (GSK-3, Akt, p70S6 kinase, p42/44 MAPK). Using

this method the quantitative evaluation of changes in their phosphorylation state in response to treatment of insulin target cells requires one single-well reaction, only, rather than seven separate immunoprecipitation reactions followed by seven immunoblotting and fluorescent/luminescent imaging procedures. Thus, this method will significantly facilitate the throughput characterization of compounds/drug candidates with insulin-like and/or insulin-sensitizing activity and concomitantly increase its accuracy and resolution.

K.6.3.4.7 Protein Interaction Analysis

PURPOSE AND RATIONALE

In addition to protein phosphorylation, protein-protein interaction represents the major molecular mechanism for insulin signal transduction in insulin target cells (see K.6.3 and K.6.3.4), as exemplified by binding to and activation by IRS of PI3-K. Protein-protein interaction can be measured by (i) coimmunoprecipitation of one partner and subsequent immunoblotting of the coimmunoprecipitate for presence of the binding partner, (ii) pull-down of one partner *via* its tag (e. g. GST, His) and subsequent immunoblotting of the recovered material for presence of the (tagged) binding partner and (iii) the optical phenomenon called surface plasmon resonance (SPR). More than one decade after its introduction (Jönsson et al. 1991), latter method (iii) combined with a miniaturized flow system and a sophisticated surface design has gained a lot of attractiveness since it enables many biomolecular interactions to be investigated conveniently in real time and does depend neither on the availability of a pair of compatible antibodies as is the case for coimmunoprecipitation (i) nor on the generation of recombinant tagged or labeled binding partners as is required for pull-down (ii) and furthermore allows kinetic and real-time rather than sole steady-state analysis of the interaction.

SPR defines the characteristics of proteins in terms of their specificity of interaction with other molecules, the rates at which they interact (association and dissociation) and their affinity, which can be determined from the level of binding at equilibrium (seen as a constant signal) as a function of sample concentration as well as from kinetic measurements. Even the formation of multimolecular complexes can be monitored.

The natural phenomenon of SPR is utilized by the Biacore protein interaction analysis system (Biacore

AB, Sweden) to deliver high-quality data in real time, without the use of labels. The SPR phenomenon occurs when polarised light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media of different refractive index, the glass of a sensor surface (high refractive index) and a buffer (low refractive index). In Biacore systems, a sensor chip and microfluidics system form flow cells through which samples flow over the sensor surface. As molecules from the injected sample bind to the immobilised molecules, an alteration in refractive index occurs that is proportional to the change in mass concentration at the surface. Using the SPR phenomenon, these changes are detected in real time and data is presented as a sensorgram (SPR response plotted against real time).

PROCEDURE

In a typical experiment one of the components (binding partner, ligand) in a biospecific pair is immobilized on the sensor chip surface (Löfas et al. 1995), and the counterpart (the analyte) which interacts with the ligand is in solution and passed over the gold film in a continuous flow. In the flow system (Sjölander and Urbaniczky 1991), samples and reagents can be introduced over the sensor surface. Automation of flow properties and sample injection gives the experiment control over temperature, flow and concentration properties as well as permitting reaction conditions on the surface to be changed easily.

The surface properties of the sensor chips are central to BiaCore technology. The surface of the gold film that is needed for SPR detection is modified to avoid protein adsorption to the detector surface itself. This is achieved by a self-assembled monolayer of alkanethiols to which dextran is coupled by epoxy chemistry (Johnsson et al. 1991, Löfas 1995). The surface properties are of great importance for the evaluation of results from real-time biomolecular interaction analysis. Unwanted interactions can be identified and to some extent compensated for by proper control surfaces in multidetection approaches (Karlsson and Ståhlberg 1995). Nordin and coworkers (2005) analyzed small molecule interactions with PK using SPR biosensors

EVALUATION

A range of methods based on BiaCore technology have been developed for different purposes over the past years which have been reviewed by Malmqvist and Karlsson (1997). Biacore's system facilitates the elucidation of disease mechanisms and the discovery of

drug candidates by characterising native and recombinant protein interactions. The system enables the definition of potential drug targets, and the development of assays to characterize the interaction of target proteins with low-molecular-weight compounds. Other applications may include the selection of protein therapeutic candidates (e.g. recombinant insulin analogs) according to their on-/off-rates of binding to the target (e.g. IR) as well as the detection and characterization of immune responses during preclinical and clinical development.

Regarding application of BiaCore technology to PK-inhibitor interaction, Nordlin and coworkers (2005) found the immobilization conditions to be critical and they developed several strategies for preserving the binding capacity for inhibitors and ATP. The composition of the assay buffer is developed for optimized kinase assay performance. The assays include kinetic characterization of inhibitor binding to PK and analysis of binding characteristics in the presence of ATP for identification of ATP-competitive binders. The effect of PK phosphorylation on the binding of ATP and inhibitors can also be investigated, as is the PK isotype specificity. Furthermore, a complementary activity assay revealed the correlation between binding to the PK and inhibition of the PK. Thus, the biosensor technology offers a useful tool in PK-targeted compound identification and drug evaluation.

K.6.3.5

***O*-Linked Glycosylation (*O*-GlcNAc) of Insulin Signaling Components**

GENERAL CONSIDERATIONS

Hyperglycemia is a consequence of type II diabetes and constitutes a self-perpetuating condition that contributes to poor metabolic control by impairing both insulin action and insulin secretion (Kruszynska and Olefsky 1996, Karam 1996, Yki-Järvinen 1992). The deleterious effects of hyperglycemia have been collectively referred to as "glucose toxicity" (Rossetti et al. 1990 and 1995, Rossetti 1996), the most prominent one of which is insulin resistance. Studies of humans and animals with spontaneous or experimentally induced insulin resistance have revealed much about the multiple pathways that can lead to insulin resistance. However, the primary events that trigger insulin resistance remain incompletely understood. Experimental induction of insulin resistance in isolated cells or tissues can be a useful approach for the identification of early events because it provides considerable experimental control.

Marshall and coworkers provided ample evidence that excessive flux of glucose through the hexosamine biosynthesis pathway (HBP) triggers various adaptive responses that restore intracellular glucose homeostasis. One such response is reduction of glucose uptake through desensitization of the insulin-responsive glucose transport system (GTS). Since the glucose-sensing/signal transductional capability of the HBP constitutes only one function of this ubiquitous pathway, this regulatory arm of the HBP has been named the hexosamine signaling pathway (HSP) (Marshall and Rumberger 2000, Marshall 2002).

In exploring the functional role of the HSP and the underlying mechanisms, glucosamine (GlcN) is often used as a pharmacological tool since it can readily undergo internalization through the GLUT1/4 and directly enter the HBP at the level of GlcN-6-P (Marshall et al. 1991a, 2004). Thus, GlcN was found to mimic glucose-induced desensitization in adipocytes and was shown to be >40 times more potent than glucose. Although it was originally thought of that glucose and GlcN were causing desensitization through a common mechanism, this premise was later questioned when actinomycin-D was found to completely block glucose-induced desensitization but not GlcN-induced desensitization (Marshall et al. 1991b, Han et al. 2003). Thus, glucose-induced desensitization is mediated through a mechanism involving changes in gene expression, whereas GlcN-induced desensitization occurs through an alternate and unidentified mechanism. In conclusion, Marshall and colleagues hypothesized that flux through the HBP serves as a glucose sensor in adipocytes, and potentially other insulin target cells, and by some unknown negative feedback loop, regulates glucose transport provoking glucose-induced insulin resistance.

PURPOSE AND RATIONALE

The studies of Hebert and coworkers (1996), Cooksey and McClain (2002) and Gazdag and coworkers (2000) demonstrated an inverse relationship between cellular UDP-GlcNAc levels and insulin action, but did not reveal a specific mechanism for the association. UDP-GlcNAc is a substrate for O-GlcNAc transferase (uridine diphospho-*N*-acetylglucosamine/polypeptide-*N*-acetylglucosaminyltransferase) that transfers single *N*-acetylglucosamine moieties to the hydroxyl group of serine and threonine residues on cellular target proteins in a dynamic and reversible process known as O-GlcNAcylation (Wells et al. 2001). The extent of cellular O-GlcNAcylation appears to be regulated, at least in part, by the cellular concentration of the UDP-

GlcNAc substrate (Kreppel and Hart 1999). McClain and coworkers (2002) recently found direct evidence that O-GlcNAc transferase plays an important role in insulin action by overexpressing O-GlcNAc transferase in skeletal muscle and fat in transgenic mice and inducing insulin resistance.

O-GlcNAcase (β -*N*-acetylglucosaminidase) is responsible for specific removal of single O-GlcNAc modifications from cellular proteins. Both O-GlcNAc transferase and O-GlcNAcase exhibit nuclear and cytoplasmic localization and are highly conserved (Dong and Hart 1994). Wells and coworkers (2001) have hypothesized that O-GlcNAcylation of proteins is a type of post-translational modification that may be analogous to PK and PP regulation of phosphorylation and thus compete for similar or adjacent phosphorylation sites in a reciprocal manner. It seems possible that, in addition to flux through the HBP, regulation of either O-GlcNAc transferase (McClain et al. 2002) or O-GlcNAcase (Vosseller et al. 2001, 2002) may influence insulin action. O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) is a potent and nontoxic inhibitor of O-GlcNAcase in various cell lines (Haltiwanger et al. 1998). Arias and coworkers (2004) found that prolonged incubation in PUGNAc results in increased protein-O-linked glycosylation and insulin resistance in rat skeletal muscle. The following assays represent model systems for the induction and analysis of glucose-induced insulin resistance which is mediated by O-GlcNAcylation and can be modified by compounds/drug candidates.

K.6.3.5.1

Induction of O-GlcNAc Modification in Adipocytes, Myocytes and Muscles

Primary Adipocytes

Isolated adipocytes are obtained from the epididymal fat pads of male Sprague-Dawley rats (180–225 g) by collagenase digestion as described above with modifications introduced by Marshall and coworkers (2005). After digestion, cells are washed three times in HEPES-buffered balanced saline solution (HBSS) consisting of 25 mM HEPES (pH 7.6), 120 mM NaCl, 0.8 mM MgSO₄, 2 mM CaCl₂, 5.4 mM KCl, 1 mM NaH₂PO₄, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% BSA. Cells are then diluted to a final concentration of 5×10^5 cells/ml (12 ml HBSS per 1 g initial fat weight). From a common pool of cells, 200-µl portions of the adipocyte suspension in 12 × 75-mm sterile polystyrene tubes are in-

cubated at 37°C in the absence or presence of insulin (10 nM final conc.) to stimulate the glucose transport system and to facilitate the subsequent uptake of GlcN. After 20–30 min, GlcN is added and adipocytes are further incubated at 37°C.

3T3-L1 Adipocytes

3T3-L1 adipocytes are cultured for 16 h in DMEM containing 4 mM glucose in the absence of serum and containing PUGNAc (100 µM). After 16 h, cells are refed with identical media for 3 h. For GlcN and/or chronic insulin/glucose treatment of cells, 3T3-L1 adipocytes are cultured for 16 h in DMEM containing 4 mM glucose in the absence of serum with 0.2% BSA and containing 5 mM GlcN/5 mM HEPES (pH 7.6) and/or insulin (1 nM). After 16 h, cells are washed twice and refed with identical media used during 16-h treatment, but lacking insulin. Cells are then washed twice with Krebs-Ringer's phosphate buffer (KRP) and stimulated in KRP with various concentrations of insulin for the indicated time at 37°C.

Myocytes

C2C12 cells are cultured to confluence in growing conditions in DMEM containing 25 mM glucose and 10% fetal bovine serum (FBS) in collagen type I-coated culture plate according to the procedure described by Fujita and coworkers (2005). When cell confluence is attained, cells are cultured for 4 days with DMEM containing 25 mM glucose, 2% horse serum, and with or without 10 mM GlcN. After 4 d, the change in cell morphology induced by GlcN is observed by an optical microscope.

Muscles

Epitrochlearis muscles are quickly excised from rats (140–160 g) anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) upon loss of pedal reflexes and then transferred into the tissue incubation medium. Muscles are incubated (18 h) in flasks containing 1.5 ml of low glucose (5.5 mM) DMEM supplemented with CaCl₂ (final conc. 2.5 mM), NaHCO₃ (final conc. 25 mM), insulin (final conc. 0.6 nM) and BSA (0.1%). Insulin is included for the initial 18 h of incubation because this prevents the increase in basal glucose transport observed with prolonged incubation of isolated skeletal muscle. Flasks are placed in a shaking water bath at 35°C and continuously gassed with 95% O₂/5% CO₂. The media are replaced with fresh DMEM every 6 h during the initial 18-h incubation. To minimize bacterial contamination during prolonged incubation,

100 µU/ml penicillin and 100 µg/ml streptomycin are added to the DMEM and Teflon air filters are used to minimize aerosol contaminants.

Following the 18-h incubation in supplemented DMEM, muscles are rinsed in KHB supplemented with 0.1% BSA, 100 µU/ml penicillin, 100 µg/ml streptomycin, 2 mM sodium pyruvate and 5 mM mannitol for two 30-min periods at 30°C for removal of glucose and DMEM. During the rinse steps, the KHB buffer is supplemented with 0, 0.6 or 12 nM insulin. Muscles treated with inhibitors of GFAT in the initial incubation steps continued to be exposed to up to 100 µM inhibitor during the subsequent rinse steps. Muscles are then rapidly blotted, trimmed, clamp frozen with aluminium tongs cooled to the temperature of liquid N₂ and stored at –80°C until further processing. Total frozen muscles are weighed, transferred to pre-chilled glass tissue grinding tubes and homogenized in ice-cold lysis buffer (1 ml/muscle) containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2.5 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na₃VO₄, 20 mM glycerol-3-phosphate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM PMSF in the absence or presence of up to 100 µM of PUGNAc. Homogenates are transferred to microfuge tubes, rotated for 1–2 h at 4°C, and then centrifuged (12,000 × g, 15 min, room temperature) to remove insoluble material. Following homogenization, processed muscle samples are used for determination of the levels of GlcNAc-6-phosphate and UDP-GlcNAc as well as the amount of O-GlcNAc-modified proteins.

K.6.3.5.2

Assay for Glutamine/Fructose-6-Phosphate Amidotransferase (GFAT)

Fluorometric Method

GFA activity is assayed according to previously described methods (Yki-Järvinen et al. 1997) with modifications. Cells corresponding to 5–15 mg protein is homogenized for 10 s on ice and sonicated for 10 bursts in a Branson 250 sonifier in 10 ml of homogenization buffer (50 mM sodium phosphate, pH 7.5, 100 mM KCl, 1 mM EDTA). The sample is then centrifuged (60,000 × g, 15 min, 4°C) and the supernatant is used for assay of GFA activity. All samples are assayed immediately after homogenization. 50 µl of homogenate is incubated (45 min, 37°C) in a reaction mix (final volume 100 µl) containing 12 mM fructose-6-phosphate, 12 mM glutamine, 40 mM sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM DTT in the ab-

sence or presence of 0.5 mM of UDP-GlcNAc. The reaction is terminated with 50 μ l of 1 M perchloric acid, vortexed, and centrifuged (15,000 \times g, 15 min, 4°C). The deproteinized supernatant (145 μ l) is then treated with 258 μ l of a 1/4 mixture of tri-N-ocetylamine/1,1,2-trifluoroethane, vortexed and centrifuged (15,000 \times g, 1 min). The aqueous phase is then filtered through a 0.2- μ m filter and 50 μ l is derivatized with an equal volume of O-phthalaldehyde (OPA) solution (4 mg OPA dissolved in 50 μ l of ethanol and added to 5 ml of 0.1 M sodium borate and 10 μ l mercaptoethanol). Immediately thereafter samples are separated over a reverse-phase C18 column (25 cm \times 4.6 mm) equilibrated with 15 mM sodium phosphate (pH 7.2), 5% acetonitril, and 5% isopropanol. Absorbance of the sample eluent is analyzed using a fluorescent detector and the peak area is integrated. OPA-derivatized GlcN6P standards are run separately to determine the retention time and to generate a standard curve for correlation of area to activity. The correlation coefficient between the concentration of GlcN6P standards and the area under the GlcN6P peak is typically 0.999 or higher. The recovery of samples spiked with GlcN6P prior to derivatization is usually more than 95%.

Radiometric Method

A highly sensitive and rapid method for the separation of radiolabeled fructose-6-phosphate from glucosamine-6-phosphate has been developed by Broschat and coworkers (2002), which can be used in a disposable column or a 96-well formate and is >10-fold more sensitive than the HPLC method. The column assay has a broad range of linearity with low variation between samples. When performed in the 96-well formate, the assay is linear with time and enzyme concentration and greatly improves the rapidity and accuracy with which GFAT activity can be measured.

Research Formate

Cells are homogenized as described for the fluorometric assay using the cytosolic fraction obtained by 100,000 \times g spin for 1 h. The final assay conditions contain 20 μ M fructose-6-phosphate (300,000 cpm), 400 μ M glutamine, 20 mM imidazole buffer (pH 6.8), 1 mM EDTA, 10 mM KCl, 1 mg/ml BSA and 1 mM DTT in a total volume of 50 μ l. All assays are conducted at room temperature. Just prior to the assay, the samples prepared in stabilizing buffer containing glucose-6-phosphate (Zhou et al. 1995) are buffer-exchanged on PD-10 columns equilibrated in the GFAT assay buffer. The reaction is stopped by dilution of the enzyme reaction with 1 ml of 10 mM NaHCO₃ (pH

3.0). Separation of GlcN-6-phosphate from substrate is performed immediately at room temperature. Unbound [¹⁴C]GlcN-6-phosphate is collected in scintillation vials and quantified by scintillation counting.

Screening Formate

Strong anion exchange resin (AG1X8, 400 mesh, formate form) is prepared prior to the assay and stored for 2 weeks at room temperature or at 4°C for longer periods. The resin is suspended in batch in a 5-volume excess of 10 mM NaHCO₃ (pH 3.0) and allowed to settle for 20 min. The fines and excess buffer are decanted, and the procedure is repeated until no fine material remained, usually three to four times. The pH of the formate buffer above the resin should be unchanged by the resin after the equilibration is complete. GFAT activity is measured in a total volume of 50 μ l in U-bottomed deep-well 96-well plates. The substrates, 20 μ M [¹⁴C]fructose-6-phosphate (75,000 cpm per well) and 400 μ M glutamine are mixed and the reaction is terminated by the addition of a 150- μ l suspension of Dowex AG1X8 (400 mesh) in 10 mM NaHCO₃, containing 50 μ l resin and 100 μ l buffer by volume. Suspension of the resin is maintained using a stir plate prior to transfer. The resin is allowed to settle for 5 min, after which a 50- μ l aliquot of the supernatant is removed using a programmable liquid transfer device and transferred to a 96-well topcount plate. After addition of 150 μ l of scintillation cocktail, the plates are sealed, shaken for 5 min and counted for radioactivity in a topcount reader.

K.6.3.5.3

Assay for O-GlcNAc Transferase

Cells corresponding to 2–5 mg protein (bicinchoninic acid method using BSA as standard) are homogenized in 6 \times volume (μ l/mg) of homogenizing buffer containing 20 mM HEPES/KOH (pH 7.0), 10 mM MgCl₂, 0.4 mM Na₃VO₄, 1 mM EDTA, 4 μ M benzamide, 0.5 mM PMSF, 1 μ g/ml pepstatin, 1 ng/ml leupeptin, 0.5 μ g/ml antipain and 250 mM sucrose (HBS) in a glass homogenizer for 30 sec on ice. The homogenate is centrifuged (100,000 \times g, 25 min, 4°C). The supernatant (cytosol) is mixed with an equal volume of 30% PEG8000 and incubated on ice for 30 min to precipitate proteins. The precipitated material is pelleted by brief centrifugation (10,000 \times g, 2 min). The supernatant is discarded and the pellet is washed once with HBS and resuspended in half the original volume of HBS. O-GlcNAc transferase is measured as described by Haltiwanger and cowork-

ers (1992) with slight modifications. Both methods derive their specificity by utilizing peptide substrates that are modelled after *O*-glucosamylation motifs of known *O*-GlcNAcylated proteins. The method recommended utilizes an Arg-tagged peptide which binds firmly to phosphocellulose paper. Reactions are conducted in 45- μ l mixtures containing 1–5 μ g of cytosolic protein in 20 mM HEPES/KOH (pH 8.0), 250 mM sucrose, 0.5 mM EDTA, 5 mM peptide substrate (TITSETPSSTTTQITKR) and 0.2 μ Ci UDP-[6-³H]-N-acetylglucosamine (25 Ci/mmol). The reactions are incubated (60 min, 25°C). At the end of the incubations, 40- μ l portions are spotted onto 1-cm² Whatman P81 ion exchange papers. The paper squares are immediately immersed in 50 mM formic acid, washed extensively with 4 \times 4 ml of 50 mM formic acid per square and then counted for radioactivity in 5 ml scintillation cocktail per square. All assays should be conducted in the linear range for time, peptide concentration and homogenate protein.

K.6.3.5.4

Measurement of GlcN-6-P Levels

For determination of the GlcN-6-P levels according to the procedure of Marshall and coworkers (2005b), adipocytes or myocytes after treatment with compounds/drug candidates are transferred to 1.5-ml microfuge tubes and washed 3 times with ice-cold, BSA-free HBSS. During the final wash, the cell volume is reduced to 150 μ l and 0.4 ml of cold perchloric acid (0.3 M) is added to the cells for 10 min. The mixture is then centrifuged (20,800 \times g, 10 min, 4°C) and the deproteinated metabolite extract (420 μ l) is transferred to a new microfuge tube. After the extract is neutralized by adding a small amount of K₂CO₃ (5 M), extracts are frozen and stored at –20°C. The concentration GlcN-6-P is measured in the extracts by HPLC as previously described (Marshall et al. 2004, 2005a) by a method that entailed derivatization of GlcN-6-P with *O*-phthalaldehyde (OPA) so that it can be detected fluorometrically (following separation by reverse phase HPLC).

K.6.3.5.5

Measurement of UDP-GlcNAc Levels

This assay is performed essentially as described previously by Robinson and coworkers (1995). To avoid degradation of UDP-GlcNAc, isolated tissue samples are frozen immediately in liquid nitrogen, and then the procedure for the extraction of UDP-GlcNAc is

carried out as quickly as possible. Frozen muscle or liver tissue (~150 mg) is homogenized at 4°C in 5 volumes of PBS. A small aliquot of tissue homogenate is used for protein assay. 100% (w/v) TCA is added to tissue homogenate (10% final conc.). Samples are mixed well and the precipitates are pelleted by centrifugation (10,000 \times g, 15 min, 4°C). To eliminate TCA from the supernatant, two volumes of diethylether are added and mixed well. After centrifugation (1,000 \times g, 3 min, room temperature), the diethylether phase is discarded. This TCA elimination step is repeated 10 times. The aqueous samples are dried up and dissolved in 30 μ l distilled water. The extracts are centrifuged (20,000 \times g, 10 min, room temperature), and high pressure liquid chromatography (HPLC) is performed on a SAX anion-exchange column (4.6 \times 250 mm, e.g. Whatman Partisil) eluted with 40 mM ammonium phosphate at a flow rate of 1 ml/min. The amount of UDP-GlcNAc in the samples is quantified by ultraviolet absorption (A_{254}), and calculated from the height of peak against standard series of UDP-GlcNAc. The retention-time of the peak of UDP-GlcNAc is 12.0 min.

K.6.3.5.6

Detection of *O*-GlcNAc-Modified Proteins

Immunological Detection

For immunological detection of *O*-GlcNAc-modified proteins according to the procedure introduced by Vosseller and coworkers (2002), whole-cell lysates for immunoblotting are prepared by scraping 3T3-L1 cells from 6-cm culture dishes in 400 μ l of 1% SDS, transferring to Eppendorf tubes, and boiling for 10 min. Laemmli sample buffer is added directly to lysates for SDS-PAGE. Lysates for immunoprecipitations are obtained by scraping cells in 1% Nonidet P-40 buffer containing 15 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, protease inhibitors, and 1 μ M PUGNAc to inhibit deglycosylation during lysis. Protein concentrations in lysates are determined by using the Bio-Rad protein reagent. The anti-*O*-GlcNAc antibody used is a mouse monoclonal IgM from ascites designated 110.6 (Comer et al. 2001). For these immunoprecipitations, antibody is bound and covalently coupled by using dimethyl pimelimidate (Pierce) to agarose-conjugated anti-IgM. For immunoblotting, secondary antibody is horseradish peroxidase-conjugated goat anti-mouse IgM (μ chain-specific). In all cases, immunoblots are developed with ECL reagent and imaged on hyperfilm (Amersham Pharmacia). Immunoprecipitations with indicated antibodies are performed

overnight at 4°C and captured with protein A/G-Sepharose (Santa Cruz Biotechnology) unless otherwise indicated, washed in RIPA buffer (1% Nonidet P-40 buffer described above, but including 0.1% SDS, 0.25% sodium deoxycholate, and not containing protease inhibitors or PUGNAc), followed by washing in TBS. Proteins are boiled off Sepharose beads in Laemmli sample buffer for SDS-PAGE with precast minigels (Bio-Rad). The separated proteins are transferred to polyvinylidene difluoride (Immobilon-P, Millipore), blocked at least 1 h in TBS containing 4% BSA and 0.1% Tween20, and probed with indicated antibodies overnight at 4°C. After binding of appropriate horseradish peroxidase-coupled secondary antibodies, ECL detection is used. In cases where densitometry is used to quantitate Western blot signals, the Flourchem imaging system and software (Alpha Innotech, CA) are used.

Enzymatic Detection

Terminally O-GlcNAc-modified membrane proteins are identified by the galactosyltransferase probe method using purified UDP-Gal-GlcNAc-Gal β -(1–4) galactosyltransferase (Gal-transferase) and UDP-[³H]galactose as described by Roquemore and coworkers (1994) with modifications introduced by Buse and coworkers (2002). Briefly, 0.5 or 1 mg of total crude membrane proteins in 50 mM HEPES (pH 7.4), 150 mM NaCl, and 0.5% CHAPS is incubated for 1 h at 37°C with the addition of 10 mM HEPES (pH 7.3), 10 mM galactose, 5 mM MnCl₂, 1 mM DTT, 25 mM 5'-AMP, 20 μ Ci/ml UDP-[6-³H]galactose (40 Ci/mmol), and 1 U/ml galactosyltransferase (previously autogalactosylated for 30 min at 37°C in 50 mM Tris/HCl, pH 7.3, 5 mM MgCl₂, 1 mM mercaptoethanol, 0.4 mM UDP-galactose, and 0.1 mg/ml aprotinin). Enzyme reactions are terminated by the addition of 10 mM EDTA. Immunoprecipitation of galactosylated membranes is described below.

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K.6.3.6

Insulin-Like Signal Transduction via Plasma Membrane Microdomains (Caveolae and Lipid Rafts)

GENERAL CONSIDERATIONS

Caveolae

Caveolae were discovered as non-coated small flask-shaped invaginations (25–100 nm) of the plasma membrane by electron microscopy of endothelial cells (Fan et al. 1983, for a review see Anderson 1993a, 1993b, 1998, 1999, 2002, Shaul and Anderson 1998). It is possible that caveolae are formed from non-invaginated lipid rafts in the presence of the principal structural caveolae protein, caveolin (Kurzchalia et al. 1994, Lisanti et al. 1994, Parton 1996, Schlegel et al. 1998, Müller 1999 and 2000, Müller and Welte 2002e).

Caveolae and the so called lipid rafts are believed to be enriched in the bulk membrane lipids, such as phosphatidylinositol, cholesterol, sphingomyelin and glycolipids, such as glycosylphosphatidylinositol and the ganglioside GM1 (for reviews see Rietveld and Simons 1998, Bickel 2002). Due to the high concentration of cholesterol and sphingomyelin, caveolae and lipid rafts are much more resistant to solubilization by detergents than are other parts of the plasma membrane and intracellular membranes (Brown and Rose 1992, Brown and London 1997 and 1998, Harder et al. 1998, Müller 2002d, Edidin 2003). The concentrations of various lipids in caveolae and rafts suggest great variations between different cell types, but also reflect the different experimental approaches used. Moreover, caveolae constitute between cell types a very variable, and usually unknown, fraction of the plasma membrane. Figures for enrichment of various components in caveolae compared to the total plasma membrane therefore reflect, to a large extent, this property of the particular cell type under investigation. In addition to the high content of cholesterol and sphingolipids, caveolae are stabilized by one or more caveolin isoforms. The caveolins are a family of 21–25-kDa integral membrane proteins that form the coat structure of caveolae (Glenney 1992, Okamoto et al. 1998, Smart et al. 1999). An unusual hairpin membrane domain results in both the amino- and carboxy-termini facing the cytoplasm. Caveolins avidly bind cholesterol, which may be the basis for their association with the liquid-ordered phase of the plasma membrane. In adipocytes caveolin-1 is the most prominent isoform (Rothberg et al. 1992) and its ablation leads to a total loss of caveolae.

Caveolae are believed to be involved in a number of cellular processes, such as receptor mediated uptake and organization of transmembrane signaling (Shaul and Anderson 1998). Caveolae and lipid rafts harbor a number of components of various intracellular signal transduction pathways including G-protein-coupled receptors, heterotrimeric G-proteins, RTK (Src family tyrosine kinases), components of the Ras-mitogen-activated protein kinase (MAPK) pathway, protein kinase C isoforms, and endothelial nitric oxide synthase (eNOS) (Schlegel et al. 1999, Shaul and Anderson 1998, Smart et al. 1999). As a consequence, they may function as locations for the direct physical interaction of signaling elements where the cross-talk between the corresponding signaling pathways takes place (Okamoto et al. 1998). Lisanti and coworkers demonstrated direct binding of a common domain within caveolin, the caveolin-scaffolding do-

main (CSD) to the corresponding caveolin-binding domain (CBD) of a variety of signaling proteins which are thereby kept in a basal inactive state, however competent for future activation (Couet et al. 1997a, Ju et al. 1997, Mineo et al. 1998, Müller 2001b, Moffett et al. 2000). The inhibitory interaction between caveolin and signaling molecules should be accessible for modulation in response to extracellular/intracellular signals. Activation of signaling pathways engaging CBD-harboring signaling molecules requires their relief from binding to/inhibition by caveolin both in short and long term (Couet et al. 1997a and b, Kobzik et al. 1996).

Lipid Rafts

Discovery of the intracellular molecules and pathways that mediate insulin action has preoccupied many investigators for many years, but controversy and confusion remain. An emerging concept to explain such signaling specificity in the face of molecular promiscuity is that specialized scaffold, anchor, and adapter proteins segregate signaling molecules into specific cellular compartments *via* protein-protein interactions (Mastick et al. 1998). One level of spatial organization proposed for the plasma membrane is that of the liquid-ordered phase. According to a model proposed by Simons and Ikonen (1997) and Simons and Toomre (2000), lipids in the liquid-ordered phase pack together to form dynamic “rafts” in the plasma membrane, and these lipid rafts either recruit or exclude specific molecules, including signaling proteins. The biophysical and biochemical microenvironment of the rafts (e. g., membrane fluidity) may influence the function of the raft proteins. Also, the physical segregation of proteins into such “microdomains” may regulate the accessibility of those proteins to regulatory or effector molecules. The structural bases suggested for protein association with rafts include glycosphosphatidylinositol (GPI) anchors (e. g., folate receptor), dual fatty acylation by myristylation and palmitoylation (e. g., Src family tyrosine kinases), transmembrane domain structure (e. g., influenza hemagglutinin polypeptide), cholesterol binding (e. g., caveolins), and protein-protein interactions (e. g., caveolin-interacting proteins). On the basis of their ability to sort proteins, rafts have been implicated in a number of cell functions, including signal transduction.

Signaling Function of Lipid Rafts and Caveolae

A pathway for insulin activation of glucose transport has been identified in insulin-sensitive cells which involves rafts/caveolae and the in- and out-movement of

signaling proteins (Baumann et al. 2000, Ribon et al. 1998). Thus, rafts/caveolae seem to harbor signaling components for insulin-stimulated glucose transport and regulate their activity by interaction with caveolin and additional caveolar structural proteins that may serve as scaffolding proteins for concentrating and activating/inhibiting specific insulin signaling components. In addition, Baumann (2001) and coworkers presented a large body of evidence that a signal generated at the lipid rafts of adipocytes in response to insulin is required for insulin-stimulated glucose transport, in addition to that elicited by the PI3-K pathway (see K.6.3.6.3).

Interestingly, compounds which manage to modulate the interaction of caveolin/caveolae with CBD-containing signaling proteins and, in consequence, the activation state of the corresponding downstream signaling cascades have been described. A study by Müller and coworkers (2001a, b) demonstrated that in isolated rat adipocytes, the non-RTK, pp59^{Lyn} and pp125^{Fak}, are down-regulated by localization in caveolae/binding to caveolin and up-regulated by release from caveolae/dissociation from caveolin in response to phosphoinositolyglycans (PIG), degradation products of GPI membrane protein anchors (Jones and Varela-Nieto 1999, Müller and Frick 1999), and the sulfonylurea drug, glimepiride (Langtry and Balfour 1998). The short term redistribution of these kinases from caveolae/rafts induces insulin-independent activation of the metabolic insulin signaling cascade. These findings suggest that the concentration of signaling proteins at caveolae/caveolin may be regulated by diverse physiological and pharmacological stimuli thereby integrating cross-talk to various signal transduction pathways.

PURPOSE AND RATIONALE

In rat adipocytes and in 3T3-L1 adipocytes the IR is localized to caveolae (Müller et al. 2002c, Gustavsson et al. 1999). The immediate downstream signaling molecule, IRS1, on the other hand, has been reported not to be associated with the plasma membrane in rat adipocytes. In rat adipocytes the disruption of caveolar integrity by cholesterol depletion using β -cyclodextrin, reversibly makes the cells insulin resistant by inhibiting the IR from phosphorylating IRS1. On the other hand, insulin signalling for mitogenic control *via* MAPK, ERK1 and 2, is not impaired in the rat cells (Gustavsson et al. 1996). The importance of caveolae for insulin action is reinforced by the finding that, in response to insulin stimulation, the insulin-regulated glucose transporter, GLUT4, is translocated

to caveolae for glucose uptake (Gustavsson et al. 1996, Kandror et al. 1995), and that some of the downstream signalling for enhanced glucose uptake may take place in caveolae.

In addition, destruction of caveolae causes a reduced uptake of long-chain fatty acids in 3T3-L1 cells and HepG2 cells (Pohl et al. 2002, Meshulam et al. 2006), and the major structural protein of caveolae, caveolin, and the caveolae-localized protein, CD36, both bind fatty acids. Caveolin has been implicated in TAG metabolism through its association with lipid bodies and LD in 3T3-L1 adipocytes and other cell lines (Fujimoto et al. 2001, Ostermeyer et al. 2001, Pol et al. 2001) and its mediation of the interaction between perilipin and the catalytic subunit of PKA which is required for β -adrenergic-dependent phosphorylation of perilipin and activation of lipolysis in adipocytes (Cohen et al. 2004). Caveolin-1^{-/-} mice that are deficient in caveolin and caveolae have, moreover, been found to have deranged lipid metabolism and atrophied adipose tissue (Drab et al. 2001, Razani et al. 2001, 2002a, for a review see Razani et al. 2002b). Furthermore, caveolin-1^{-/-} mice show a 90% decrease in IR expression selectively in adipose tissue, greatly reduced insulin-induced phosphorylation of PKB, and insulin resistance (Capozza et al. 2005, Oshikawa et al. 2004, for a review see Ishikawa et al. 2005). These findings indicate that intact caveolae are important for normal insulin signalling and (insulin) regulation of metabolism of carbohydrates and lipids in adipocytes. The following methods enable the analysis of the role of rafts/caveolae in insulin and insulin-like signal transduction in adipocytes as well as of their participation in the insulin-like activity of compounds/drug candidates.

K.6.3.6.1

Preparation of Plasma Membranes

Lipid rafts are routinely prepared from the plasma membranes of differentiated mammalian cells, preferably primary and cultured adipocytes or lung endothelial cells, due to their relative abundance.

K.6.3.6.1.1

Sucrose Gradient-Based Method

Basal and insulin/compound-stimulated adipocytes are homogenized in 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 0.25 M sucrose, 25 mM NaF, 1 mM pyrophosphate with protease inhibitors (10 μ M leupeptin, 1 μ M pepstatin, 1 μ M aprotinin, 4 mM iodoacetate and 50 mM PMSF) using a motor-

driven Teflon-in-glass homogenizer at room temperature. Subsequent procedures are carried out at 4°C. Cell debris and nuclei are removed by centrifugation at (1,000 \times g, 10 min). A plasma membrane-containing pellet, obtained by centrifugation (16,000 \times g, 20 min) is resuspended in 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and protease inhibitors. The plasma membranes are purified by sucrose density gradient centrifugation (Avruch and Wallach 1971).

K.6.3.6.1.2

OptiPrep-Step Gradient-Based Method

Purification of plasma membranes from total membranes is performed by fractionation using the OptiPrep gradient (AXIS-Shield) according to the procedure of Pohl and coworkers (2005). Monolayers of confluent 3T3-L1 adipocytes are washed once in PBS to remove the culture medium and then once in the homogenization buffer (10 mM Tris/HCl, pH 7.4, 25 mM sucrose, 0.5 mM EDTA). Thereafter, cells are scraped in 3 ml of homogenization buffer and centrifuged (1,000 \times g, 5 min). The pellet is resuspended in 2 ml of homogenization buffer. Cells are lysed by 10 passages through a fine syringe needle followed by treatment with a tight-fitting Dounce homogenisator. The homogenate is centrifuged (1,000 \times g, 10 min). The resulting post-nuclear supernatant is centrifuged (100,000 \times g, 40 min). The resulting total membrane pellet is resuspended in 1 ml of homogenization buffer containing 25% (w/v) iodixanol and loaded onto a nine-step OptiPrep gradient, which consisted of 25, 22, 19, 16, 13, 10, 7, 4, and 1% (w/v) iodixanol. After centrifugation (200,000 \times g, 3 h, 4°C, Beckman SW41Ti), 18 fractions are collected from the bottom of each centrifuge tube. A portion of each fraction can be analyzed by SDS-PAGE and immunoblotting for markers of the plasma membranes. Fractions 5, 6 and 7 are used as purified plasma membranes according to their enrichment of Na⁺/K⁺-ATPase, CD36 and caveolin-1.

K.6.3.6.1.3

Affinity Partitioning-Based Method

All work including the two-phase systems is done at 4°C in a well-tempered cold room as two-phase partitioning is critically dependent on temperature. Male Sprague-Dawley rats, weighing 200–300 g, are starved overnight and killed by decapitation. Lungs are excised and thoroughly perfused with ice-cold homogenization medium (0.25 M sucrose, 5 mM Tris/HCl, pH 8.0). They are cut into pieces and squashed in a garlic press. Four grams of lungs are transferred to a Potter-Elvehjem homogenizer containing a 30-g

two-phase system including the lung tissue. The two-phase system consisted of 5.7% (w/w) dextran T500 (bottom phase) and 5.7% (w/w) PEG 3350 (top phase) in 15 mM Tris/H₂SO₄ (pH 7.8) (final concentrations in the system). Homogenization is done by 5 up and down strokes by a motor-driven homogenizer with a Teflon pestle at 1,000 rpm. The homogenate is transferred to a Dounce homogenizer and further homogenized by 10 strokes with a loose-fitting pestle followed by 10 strokes with a tight-fitting one. After homogenization, the phases are separated by gentle centrifugation (270 × *g*, 5 min, Sorvall SS-34 rotor). The PEG-rich top phase is collected and the dextran-rich bottom phase re-extracted with fresh top phase by mixing and phase separation as above. The two top phases collected are combined and further subjected to affinity partitioning. The fresh top phase for re-extraction is obtained from a similar 30-g two-phase system as above, replacing the lung tissue with 4 g of 15 mM Tris/H₂SO₄ (pH 7.8). After weighing in the components, the system is thoroughly mixed and allowed to settle overnight before collecting the top phase to be used for re-extraction. The phases for affinity partitioning are obtained as follows: a 20-g system containing 6.0% (w/w) PEG 3350 and 6.0% (w/w) dextran T500, including 1.3 mg WGA coupled to dextran T500, in 15 mM borate/Tris (pH 7.8) (final concentrations), is weighed in. After thoroughly mixing the system and letting settle overnight, the phases are collected individually, the WGA-dextran-containing bottom phase to be used for the initial affinity partitioning step and the PEG-enriched top phase for re-extraction (Persson et al. 2001). The combined top phases from the partitioning step above are mixed thoroughly with the WGA-containing bottom phase by 20 inversions, vortexing, and another 20 inversions. After phase separation through gentle centrifugation as above, the top phase is removed and the bottom phase re-extracted in the same manner with the fresh top phase obtained from the pre-equilibrated 20-g system. The final bottom phase is collected, diluted 10-times with 0.1 M *N*-acetylglucosamine in 5 mM Tris/HCl (pH 8.0) to dissociate membranes from WGA-dextran, and centrifuged (100,000 × *g*, 90 min). The plasma membrane pellet is resuspended in homogenization medium.

K.6.3.6.2

Preparation of Lipid Rafts and Caveolae

PURPOSE AND RATIONALE

Typical preparations of lipid rafts use 1% Triton X-100 to extract whole cells and the low-density, deter-

gent-resistant material is separated from other solubilized membrane fractions by centrifugation on a 5% to 30% sucrose gradient (Brown and Rose 1992). Subsequently, lipid rafts have been prepared using a variety of other detergents, including Lubrol WX, Lubrol PX, Brij58, Brij96, Brji98, Nonidet P40, CHAPS, and octylglucoside (for a review see Macdonald and Pike 2005). Although preparations of detergent-resistant membranes are readily isolated, several observations have raised concerns that extraction of cells with detergent may be generating clusters of raft lipids and proteins that did not exist in the intact cell. For example, examination of cells grown on coverslips and then extracted with Triton X-100 reveals a continuous membrane sheet pock-marked by large holes (Major and Maxfield 1995). Because rafts are thought to be < 50 nm in diameter (Anderson and Jacobson 2002, Edidin 2003), the residual “detergent-resistant” membranes probably form from individual rafts that coalesced as a result of the detergent treatment.

To avoid the complications associated with preparing rafts using detergent extraction procedures, several methods have been established for isolating rafts from cells fractionated in the absence of detergent. Song and coworkers (1996) sonicated cells in a pH 11 sodium carbonate buffer and isolated caveolae by centrifugation of the lysate in a discontinuous 5%/35%/45% sucrose gradient. Although this procedure is relatively easy, the spin time of 16–20 h is long and the resulting raft fraction is significantly contaminated with other membranes. A more careful fractionation of lipid rafts (Smart et al. 1995), which is based on lysis of the cells in an isotonic buffer containing EDTA and purified plasma membranes on a Percoll gradient. The plasma membranes are then sonicated and the lipid rafts isolated by flotation through a 10–20% gradient OptiPrep. This method results in the production of a relatively clean raft preparation. However, it is time-consuming and yields are poor. Furthermore, there is significant variability from preparation to preparation and from cell type to cell type. Because of the drawbacks associated with those procedures for isolating detergent-free lipid rafts, Macdonald and Pike (2005) developed an improved method that allows the rapid isolation of purified rafts in good yield. It is based on lysis of cells by shearing in an isotonic buffer containing calcium and magnesium and apparently allows isolation of a highly purified raft fraction in a single step by flotation in a 0–20% OptiPrep gradient.

Studies on caveolae structure and function would be facilitated by a convenient high-yield isolation procedure. Most isolation protocols developed involve the

detachment of caveolae from crude membranes either by Triton X-100 treatment (Sargiocomo et al. 1993, Chang et al. 1994) or by sonication (Smart et al. 1995) followed by density gradient centrifugation in either sucrose (Sargiocomo et al. 1993, Chang et al. 1994) or OptiPrep gradients (Smart et al. 1995). To obtain highly purified caveolae, however, extensive purification of a suitable plasma membrane fraction is required prior to detachment and centrifugation as was demonstrated using lung endothelial cells (Schnitzer et al. 1995a, b). Alternatively, immunoisolation protocols may be used (Oh and Schnitzer 1999, Abedin-pour and Jergil 2003), although these also require pre-purification steps.

PROCEDURE

Conventional plasma membrane purification protocols based on centrifugation techniques usually result in low yields. In an alternative approach rat liver plasma membranes are purified by affinity partitioning in a PEG–dextran aqueous two-phase system (see above). Partitioning conditions are chosen to direct the bulk of unfractionated membranes to the PEG-rich top phase and then selectively to attract plasma membranes to the dextran-rich bottom phase by including WGA conjugated to dextran as an affinity ligand. This lectin specifically binds to *N*-acetylglucosamine and sialic acid residues on the plasma membrane surface. The affinity purification method is rapid and resulted in plasma membranes of high purity and yield (Persson et al. 1991, Persson and Jergil 1992). The possibility of combining two-phase affinity purification of plasma membranes with density gradient centrifugation to isolate caveolae has been explored (Abedin-pour and Jergil 2003). Such a method should be generally applicable for the preparation of caveolae from various tissues and cells. The isolation of a vesicular fraction from rat lung highly purified in caveolin, a caveolar marker protein, by this approach is reported.

K.6.3.6.2.1

Carbonate-Based Method

Purified plasma membranes are pelleted and resuspended in 0.5 M Na₂CO₃ (pH 11), and sonicated with a probe-type sonifier 3 × 20 s. The homogenate is then adjusted to 45% (w/v) sucrose in 12 mM MES (pH 6.5), 75 mM NaCl, 0.25 M Na₂CO₃, with protease inhibitors, and loaded under a 5–35% (w/v) discontinuous sucrose gradient in the same solution and centrifuged (39,000 rpm, 16–20 h, 4°C, Beckman SW41 rotor). The light-scattering band at the 5–35% sucrose interface is collected and referred to as stan-

dard caveolae fraction. The recovery of caveolin in the caveolae fraction is typically 25 ± 5% as determined by immunoblotting. Alternatively, for preparation from intact adipocytes, washed cells (0.5 × 10⁷ cells) are suspended in 2 ml of sodium carbonate buffer (0.5 M Na₂CO₃, pH 11) containing protease inhibitors and homogenized sequentially using a loosely fitting Dounce homogenizer (10 strokes) and a sonicator (3 × 20 s bursts). The homogenate (2 ml) is then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose, 50 mM MES/KOH (pH 6.5), 150 mM NaCl (final pH of the mixture 10.2). A discontinuous sucrose gradient is formed by overlaying this solution with 4 ml of 35% sucrose and 4 ml of 5% sucrose, both in the same buffer containing 0.25 M Na₂CO₃. After centrifugation (see above), 0.85-ml gradient fractions are collected to yield a total of 14 fractions. The individual gradient fractions are pooled into rafts (Fr. 4–7) and non-raft areas (Fr. 10–14). The membranes from each pooled gradient fractions obtained by either method are diluted 2- to 3-fold with 25 mM MES (pH 6.5), 150 mM NaCl, 1% TX-100, collected by centrifugation (50,000 × g, 30 min, 4°C) and resuspended in non-dissociating buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF and protease inhibitors) or dissociating buffer (= non-dissociating buffer supplemented with 60 mM octylthioglucoside, 0.3% deoxycholate) as indicated, incubated (1 h, on ice) and used for (co)immunoprecipitation, immunoblotting or photoaffinity labeling.

K.6.3.6.2.2

Detergent-Based Method

For preparation from intact adipocytes, washed adipocytes (3.5 × 10⁷) are suspended in 1.5 ml of lysis buffer (25 mM MES, pH 6.0, 150 mM NaCl, 5 mM EDTA, 1% TX-100, 0.2 mM sodium orthovanadate, and protease inhibitors) and incubated for 20 min on ice. The cells are lysed with 10 strokes in a manual Teflon-in-glass homogenizer over the course of 1 h at 4°C. The lysate is centrifuged (1,300 × g, 5 min) to pellet unbroken cells, cellular debris, nuclei and large insoluble material. One ml of the postnuclear supernatant is subjected to sucrose gradient centrifugation by mixing with an equal volume of 85% sucrose, 25 mM MES (pH 6.0), 150 mM NaCl, 5 mM EDTA at the bottom of a 12-ml centrifuge tube which is overlaid with 5.5 ml of 35% sucrose, and then 3.5 ml of 5% sucrose in the same medium. After centrifugation (230,000 × g, 18 h, 4°C, Beckman SW41 rotor), 0.9-ml

fractions are collected from top to bottom, and termed fraction Fr. 1, 2, 3, etc. The bottom fraction is Fr. 12. Fr. 5 appears as a white, light-scattering band under illumination located at 5–7% sucrose at the 35% sucrose interface. The rafts contained in Fr. 5 are pelleted by dilution of the sucrose with 5 volumes of 50 mM HEPES/KOH containing protease inhibitors and centrifugation ($200,000 \times g$, 2 h).

Alternatively, for preparation from plasma membrane fractions, Triton X-100 is added to the purified plasma membrane fraction to a final concentration of 1% (w/v) (approximately a 100-fold excess of detergent to membrane protein). The sample is incubated for 30 min at 4°C and centrifuged ($100,000 \times g$, 90 min). The pellet is resuspended in homogenization medium by 10 strokes in a tight-fitting Dounce homogenizer. The resulting sample is transferred to a Beckman SW40 ultracentrifuge tube. The sample is adjusted to 40% (w/v) sucrose by addition of 80% (w/v) sucrose, before layering 5.5 ml each of 24 and 5% (w/v) sucrose on top of the sample. All sucrose solutions are made in 5 mM Tris/HCl (pH 8.0). After centrifugation ($100,000 \times g$, 21 h, 4°C, Beckman SW41 rotor), 12 1-ml fractions are collected by upward displacement. The light-scattering band at the 5–35% (w/v) sucrose interface is collected as lipid rafts. The recovery of caveolin is typically 15% as determined by immunoblotting.

K.6.3.6.2.3

Immunopurification

Lipid rafts prepared from rat adipocytes (originating from 500 μ l of packed cell volume) are resuspended in 0.5 ml of homogenisation buffer (buffer H). 1% Triton X-100, 2% CHAPS, 60 mM D-octyl glycoside (final concentrations) are added to 50 μ l of resuspended rafts to a final volume of 100 μ l. After 1 h on ice, the fractions are centrifuged ($104,000 \times g$, 1 h). The pellets containing detergent resistant rafts are resuspended in 100 μ l buffer H and analyzed. For immunoprecipitations the rafts (corresponding to 750 μ l of packed cell volume) and resistant to 1% Triton X-100 treatment are suspended in 300 μ l buffer H and incubated for 4 h with 6 μ l caveolin-1 antibody supplemented with 10 μ l of packed Protein A-Sepharose during the last hour. Non-bound material is removed and the Sepharose gel is washed five times in 600 μ l buffer H. The bound proteins are eluted by boiling for 15 min in buffer H supplemented with 0.6% $C_{13}E_{12}$, 48 mM NaF, 48 mM NaCl, 5% SDS, 2% β -mercaptoethanol and 5% glycerol. For GLUT-4 analysis, rafts (corresponding to 500 μ l packed cell volume) are treated with 1% $C_{13}E_{12}$

and centrifuged ($7000 \times g$, 10 min). The supernatant is incubated for 4 h with 4 μ l caveolin-1 antibody supplemented with 7 μ l packed protein A-Sepharose during the last hour. The gel is washed and eluted as described above. Each step in the immunoprecipitation procedures is performed at 4°C. The recovery of immunopurified lipid rafts is normalized by homologous immunoblotting with anti-caveolin antibodies of the same blot following stripping of the membrane.

EVALUATION

A generally applicable method for the preparation of highly purified caveolae and/or lipid rafts based on a combination of affinity preparation of plasma membranes and density gradient centrifugation has been developed. To test this approach lung tissue have been chosen as endothelial cells lining vascular ducts are rich in caveolae. In earlier studies caveolae were prepared from plasma membranes of these cells isolated by an in situ silica-coating procedure (Schnitzer et al. 1995b). Alternatively, a crude lung homogenate can be used as starting material, preparing a plasma membrane-enriched fraction by affinity partitioning (Abedinpour and Jergil 2003). To improve purity the affinity step is preceded by homogenization of the tissue directly in a conventional aqueous two-phase system followed by phase separation to remove material disturbing the affinity step. A similar approach was elaborated successfully for the purification of plasma membranes from liver (Persson and Jergil 1992, Ekblad and Jergil 2001), suggesting that the method might be applicable also to other tissues. Lung membranes have been shown similarly to liver membranes both in the conventional and in the affinity two-phase step. The overall yield of the plasma membrane marker enzyme, 5'-nucleotidase, is somewhat lower from lung (30–35%) than from liver (50–70%), however. This is primarily due to a low recovery of 5'-nucleotidase in the affinity step rather than an unfavorable distribution of the enzyme in this step or the preceding conventional two-phase step. The enrichment of 5'-nucleotidase (8-fold) compares favorably with the 5- to 10-fold enrichment of various plasma membrane markers in the earlier isolation procedure involving silica coating of lung endothelial plasma membranes. The silica coating procedure isolates the luminal part of the plasma membrane (Schnitzer et al. 1995b), while all regions of the plasma membrane are isolated by the affinity partitioning procedure (Persson et al. 1991, Persson and Jergil 1992, Ekblad and Jergil 2001).

The plasma membrane fraction obtained by the affinity procedure is suitable for the isolation of a frac-

tion highly enriched in the caveolar marker, caveolin, after dispersion of the membranes in Triton X-100 and floatation in a sucrose density gradient. The enrichment of caveolin is approximately 20-fold in this density gradient step, slightly higher than the 13-fold enrichment observed when caveolae-rich vesicles are isolated from silica-coated endothelial plasma membranes (Schnitzer et al. 1995b). However, both the affinity partitioning method presented here and the silica coating procedure result in plasma membrane material suitable for obtaining highly purified caveolae by sucrose gradient centrifugation regardless of the enrichment of caveolin attained.

Caveolae may be isolated also by a detergent-free method, gradient centrifugation by sonication of affinity purified plasma membranes. Although the yield is much lower, this nevertheless shows that the separation also works for the detergent-free preparation of caveolae-enriched material. It may be possible to optimize shearing of the plasma membrane preparation and the gradient separation to increase the yield, particularly since much vesicular material containing caveolin remained in the high-density part of the gradient. The caveolin-enriched fraction isolated after Triton X-100 treatment consists of morphologically similar closed vesicles of the same size (50–100 nm) as that of caveolae previously isolated from lung endothelial cells (Schnitzer et al. 1995a and 1995b, Stan et al. 1997). Notably no vesicles in the size range 200–700 nm are observed. Such vesicles are common in Triton-insoluble membranes prepared from crude lung homogenates, being either non-caveolar membranes or membranes occasionally having attached caveolae (Schnitzer et al. 1995d). They are not present, however, in caveolar preparations obtained by Triton X-100 treatment of endothelial plasma membranes isolated by the silica coating procedure followed by sucrose gradient centrifugation. The thin-section micrographs indicated that many of the isolated vesicles are not smoothly rounded but had an irregular shape.

K.6.3.6.3

Lipid Raft- and Caveolae-Based Assays in Insulin and Insulin-Like Signal Transduction

PURPOSE AND RATIONALE

For studying signal transduction processes which involve caveolae, these structures are isolated as rafts by biochemical methods (carbonate or detergent extraction) from primary or cultured differentiated cells, such as muscle and fat cells, after incubation with external stimuli. Rafts can be prepared either directly

from total cells, or total cell lysates or (preferably) isolated (purified) plasma membranes. The use of rafts from total cells or lysates may obscure subtle changes in signaling since the (considerable) fraction of rafts present in membranes of the Golgi apparatus (where their biogenesis takes place) may respond differently (or not at all) toward external stimuli compared to the plasma membrane rafts. Subsequent analysis for the presence in/cofractionation with caveolae, direct interaction with caveolin and changes in activity of signaling proteins in response to the external stimuli requires the methods of enrichment/purification of rafts, photoaffinity labeling (of GPI proteins), coimmunoprecipitation with caveolin (of signaling proteins), immunoblotting (of signaling proteins) and immune complex kinase assays (in case of kinase activity of signaling proteins).

K.6.3.6.3.1

Co-Immunoprecipitation of Lipid Raft Proteins

Total cell lysates (25–50 µg protein) or rafts in non-dissociating buffer (10–15 µg protein) are pre-cleared (1 h, 4°C) with protein G/A-Sepharose (50 mg/ml) in a total volume of 100 µl and then supplemented with appropriate antibodies (e. g. pp125^{Fak}, 2 µg/sample; IRS-1, 1:50 dilution; IRS-2, 10 µg/sample; pp59^{Lyn}, 5 µg/sample; caveolin, 0.7 µg/sample; IRβ, 1:175 dilution) pre-adsorbed on protein G-Sepharose (monoclonal antibodies) or protein A-Sepharose (rabbit antibodies) in a total volume of 100 µl. After incubation (4 h, 4°C, end-over-end rotation) and centrifugation (3,000 × g, 2 min, 4°C), the collected immune complexes are washed twice with 1 ml each of immunoprecipitation buffer (50 mM HEPES/KOH, pH 7.4, 500 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate) containing 0.2% Nonidet P-40 and 0.3% deoxycholate, then twice with 1 ml each of immunoprecipitation buffer containing 150 mM NaCl and 0.2% Nonidet P-40 and once with 1 ml of immunoprecipitation buffer lacking salt and detergent and finally suspended in 50 µl of Laemmli sample buffer (4% SDS, 115 mM Tris/HCl, pH 6.8, 1 mM EDTA, 10% glycerol, 4 mg/ml bromophenol blue) supplemented with 1.2% β-mercaptoethanol (except for anti-caveolin immunoprecipitates), heated (95°C, 2 min) and centrifuged. The supernatant samples are analyzed by SDS-PAGE (4–12% Bis-Tris precast gel, pH 6.4, MES/SDS running buffer under reducing conditions) and subsequent immunoblotting (see K.6.3.4.3 and K.6.3.6.3.2). The centrifugation conditions for collection of the protein A/G-Sepharose-bound immune

complexes, do not lead to sedimentation of (non-dissociated rafts) to any significant degree according to immunoblotting with anti-caveolin antibody. The coimmunoprecipitation of proteins with caveolin from non-dissociated rafts is specific for raft-associated components, such as the GPI protein, Gce1, and the dual-acylated non-RTK, pp59^{Lyn}, since the use of non-immune IgG instead of anti-caveolin antibody or of dissociated DIGs and anti-caveolin antibody do not result in immunoprecipitation of Gce1 and pp59^{Lyn}.

K.6.3.6.3.2

Immunoblotting of Lipid Raft Proteins

Immunoblotting is performed as described (see K.6.3.1.3.2.1 and K.6.3.4.3) with minor modifications. After SDS-PAGE and transfer of the proteins to PVDF membranes (2 h, 400 mA in 20% methanol, 192 mM glycine, 25 mM Tris, 0.005% SDS), the blocked membrane (1 h in 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 0.1% Brij, 0.01% NP-40 and with 1% ovalbumin and 1% BSA (anti-phosphotyrosine, anti-pp59^{Lyn}, anti-IR β) or with 5% non-fat dried milk (anti-caveolin, anti-IRS-1/2) is incubated (2 h, 25°C) with antibodies against IRS-1 (1:500), IRS-2 (1:750), caveolin (1:2,000), pp59^{Lyn} (1 μ g/ml) or IR β (2 μ g/ml) diluted in the same medium, and then washed five times with the same medium. After incubation of the membranes (1 h, 25°C) with horseradish peroxidase-coupled goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody diluted in the appropriate blocking buffer (1:5,000 or 1:2,500) and subsequent washing with detergent-containing (two times) and detergent-free (two times) buffer (20 mM Tris/HCl, pH 7.6, 150 mM NaCl), the labeled proteins are visualized by the enhanced chemiluminescence method.

K.6.3.6.3.3

Immunocomplex Assay for Lipid Raft PK

Immunocomplex kinase assays are performed as described (see K.6.2.9.2 and K.6.3.4.4) with minor modifications. Immune complexes are suspended 30 μ l of kinase buffer (50 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 1.25 mM MnCl₂, 12.5 mM MgCl₂, 1.25 mM EGTA, 0.5 mM DTT, 1 mM Na₃VO₄) containing [γ -³²P]ATP (final conc. 40 μ M, 0.2 mCi/ml) or 1 mM ATP and incubated (e. g. for pp59^{Lyn}: 15 min, 22°C) in the presence of 1 μ g heat-denatured enolase. Phosphorylation is terminated by addition of 10 μ l of four-fold concentrated Laemmli buffer and boiling. The phosphoproteins are separated on SDS-PAGE (10% Bis-Tris resolving gel, MOPS/SDS running buffer) and analyzed for phosphotyrosine by phospho-

rimaging ([γ -³²P]ATP) or immunoblotting (ATP). Under these conditions the kinase reactions are linear with time for the assay period. IRS-1 immune complexes are assayed for PI3-K by incubating (10 min, 22°C) in 50 μ l of 20 mM Tris/HCl (pH 7.0), 50 μ M [γ -³³P]ATP (5 μ Ci), 10 mM MgCl₂, 2 mM MnCl₂, 100 mM NaCl, 2 mM EDTA, 0.5 μ M wortmannin (for control incubations, only) containing 10 μ g of phosphatidylinositol (PI) and 1 μ g of phosphatidylserine. After addition of 10 μ l of 8 M HCl and 160 μ l of a 1/1 mixture of methanol/chloroform, the extracted phospholipids are resolved by TLC on plates coated with 1% oxalate and developed in chloroform/methanol/water/ammonia (60/47/11.3/3.2, by vol.). Radiolabeled PI3-P is quantitated by phosphorimage analysis. For calculation of wortmannin-sensitive PI3-K, all values are corrected for PI3-P radiolabeled in the presence of wortmannin. SDS-PAGE and subsequent immunoblotting for caveolin and the PK assumed to localize permanently or transiently at lipid rafts (e. g. protein kinase C) is performed as described (K.6.3.6.3.2).

K.6.3.6.3.4

Electron Microscopic Analysis of Caveolae

PURPOSE AND RATIONALE

Several laboratories have localized important elements of the insulin signal transduction machinery to caveolae or rafts, including the IR itself. Baumann and coworkers (2000) and Chiang and coworkers (2001) have reported the discovery of a novel insulin-signaling pathway in adipocytes that relies on proper localization of signaling molecules to lipid rafts and that is required for insulin-stimulated glucose uptake. Using a detergent-based method of lipid raft preparation, Mastick and coworkers (1995) found only trace levels of insulin receptor in the rafts of 3T3-L1 adipocytes. Müller and coworkers (2001a, b) characterized the composition of rafts from isolated rat adipocytes. The rafts and non-rafts were prepared by both detergent and detergent-free methods from total cell lysates and from plasma membranes, respectively. Representative fractions were analyzed by immunoblotting for known raft and nonraft proteins. Despite the methodological differences, there was good agreement between the proteins enriched in rafts and non-rafts by the two procedures. By both methods, the β -subunit of the IR was relatively depleted from the rafts and relatively enriched in the non-rafts. However, Gustavsson and coworkers (1999) reached the opposite result by double immunogold transmission EM and detergent-free isolation of rafts. In these experiments, caveolin and

both subunits of the IR colocalized in ~50-nm-round structures that corresponded to individual caveolae and in clusters of these structures. This colocalization was confirmed by immunofluorescence microscopy in paraformaldehyde-fixed 3T3-L1 adipocyte plasma membrane sheets. The immunofluorescence labeling pattern appeared as doughnut-like spots of 0.3–0.5 μ m diameter, a size that was noted to be similar to that of the caveolae clusters visualized by EM. Biochemically, the IR cofractionated with caveolin in detergent-free isolation of rafts from rat adipocyte plasma membranes. The addition of insulin was not associated with a significant change in localization of the IR, as assessed biochemically or by immunofluorescence. Even low concentrations of Triton X-100 (0.1%) completely solubilized the β -subunit of the IR from the caveolin-enriched fractions. This observation may explain why Mastick and coworkers (1998) found only trace amounts of the IR in rafts prepared on the basis of detergent insolubility. The molecular basis for the (partial or transient) localization of IR at lipid rafts may rely on the interaction of IR with caveolin-1, which is assumed to differentially modulate insulin signaling, as has been demonstrated by Nystrom and coworkers (1999).

PROCEDURE

Samples of appropriate fractions from the sucrose gradient are mixed with 10 volumes of 3% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 (final conc.). The sample is incubated (40 min, 4°C) followed by centrifugation (100,000 \times g, 90 min). The pellet is washed twice with 0.1 M sodium cacodylate (pH 7.4), treated with 1% OsO₄ for 1 h, and washed twice with 0.1 M sodium cacodylate. Samples were stepwise dehydrated in ethanol, treated with 1% uranyl acetate in ethanol, and embedded in Epon. Thin sections (50 nm) were stained with uranyl acetate and lead by standard procedures and examined and micrographed in an electron microscope.

Samples of the caveolae-enriched fractions are also examined following negative staining after fixation with 2% paraformaldehyde. The material is attached to carbon and pioloform-coated nickel grids for 20 min. The grids are washed three times for 5 min with phosphate-buffered saline before staining with 1% uranyl acetate for 2 min and micrographing in an electron microscope.

EVALUATION

An insulin-independent pathway has been described that mimics the metabolic actions of insulin in

adipocytes (Müller et al. 2001a, b). This pathway can be stimulated in adipocytes by exogenous phosphoinositolglycans (PIG), cleavage products of the glycosyl-phosphatidylinositol (GPI) anchor of GPI-linked proteins, which have initially been proposed to act as soluble mediators of insulin action, but do not share structural and functional similarity with the typical second messengers regulating glycogen metabolism in response to adrenergic hormones (Wasner et al. 2003). PIG treatment of isolated rat adipocytes leads to concentration-dependent increases in tyrosine phosphorylation of IRS-1, IRS-1-associated PI3-K activity, and glucose uptake in an insulin-independent fashion. A critical mechanism in this pathway may involve the dissociation of the non-RTK, Lyn and Fak, from caveolin-1 (Müller et al. 2000), their activation by tyrosine phosphorylation, and their redistribution from rafts to non-raft membranes (Müller et al. 2001b, Müller et al. 2005). Incubation of adipocytes with PIG, as with insulin, is followed by tyrosine phosphorylation of caveolin-1. The same research group succeeded in the characterization and identification of a specific binding protein for synthetic PIG in the lipid rafts of the adipocyte plasma membrane (Müller et al. 2002a, b). The involvement of lipid rafts in insulin-mimetic signaling by PIG was demonstrated by complete abrogation of redistribution of signaling proteins and IRS-1 tyrosine phosphorylation in response to PIG challenge of rat adipocytes, which have been depleted of lipid rafts by prior cholesterol removal from the plasma membrane (Müller et al. 2002e). The physiological role of this pathway and its potential cross talk with the insulin-signaling pathway in caveolae remain to be determined (Müller 2002d). These questions will be facilitated by molecular cloning and inactivation or overexpression of the gene coding for this PIG receptor in 3T3-L1 adipocytes.

Several laboratories have investigated whether there is overlap between membrane compartments that contain caveolin and GLUT4. Using biochemical isolation techniques, Scherer and coworkers (1994) detected a transient increase in GLUT4 immunoreactivity in rafts after insulin stimulation of 3T3-L1 adipocytes. Also, insulin stimulation led to an increase in plasma membrane caveolin and a corresponding decrease in caveolin detected in low-density microsomes. These investigators detected caveolin in GLUT4 vesicles immunisolated from rat adipocytes. Kandror and coworkers (1995) conducted similar studies but arrived at a different conclusion. They found that intracellular caveolin and GLUT4 were detectable on vesicles of similar size and buoyant density and

that these vesicles translocated to the plasma membrane in response to insulin. However, in contrast to the findings of Scherer and coworkers (1994), only limited amounts of caveolin protein were present in immunoabsorbed GLUT4 vesicles, which suggested that the intracellular pools of these proteins, although biophysically similar, were distinct. Gustavsson and coworkers (1996) isolated subcellular membrane fractions from rat adipocytes and performed glucose uptake assays on intact adipocytes at various times during an insulin pulse. On basis of the results obtained, the authors propose a model such that, upon insulin stimulation, GLUT4 moves first to the PM and then to detergent-resistant plasma membrane microdomains (caveolae?), where GLUT4-mediated glucose transport occurs. Taken together, at this point the weight of the published data supports localization of a significant subset of plasma membrane IR and GLUT4 to caveolae in adipocytes, but the issue is not settled (for a review see Bickel 2002).

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- 1, alkaline phosphatase and CD16 receptors, and GPI-PLC have been found in human neutrophils, bovine brain, rat intestine and a human carcinoma cell line. The GPI anchor consisting of three mannose residues and a non-acetylated glucosamine, one end of which amide-linked to the protein moiety *via* a phosphoethanolamine bridge and the other end glycosidically linked to the 6-hydroxyl group of PI, is cleaved by (G)PI-PLC/D releasing diacylglycerol or phosphatidic acid, respectively, and leaving a terminal inositolglycan structure at the protein moiety harbouring (PIG) or lacking a (cyclic) phosphate residue (for reviews see Chan et al. 1988, Cross 1990, Ferguson 1991, Müller 2000).

In eukaryotes, some GPI-PLC/D are up-regulated by glucose as well as certain hormones, growth factors and drugs (e.g. insulin, glimepiride) in rodent adipocytes, myocytes and human endothelial cells (Müller et al. 1993, 1994a, 1994c, Mohavedi and Hooper 1997). In particular, the antidiabetic drug, glimepiride, which lowers blood glucose predominantly by stimulation of insulin release from pancreatic β -cells and, in addition, to a minor degree by mimicking metabolic insulin action in peripheral tissues, such as activation of glucose transport in muscle cells and inhibition of lipolysis in adipocytes (Müller et al. 1994d, Bähr et al. 1995), has been demonstrated to activate a GPI-PLC. Upon treatment of primary or cultured rodent adipocytes with pharmacological concentrations of this sulfonylurea, a potent amphiphilic-to-hydrophilic conversion of a subset of GPI-proteins by activation of a GPI-PLC has been observed (Müller et al. 1993, 1994b). The functional and physiological significance of the insulin- and glimepiride-regulated lipolytic cleavage of GPI-proteins in mammalian cells is not well understood at present, albeit initially a role as soluble mediators for metabolic insulin action has been proposed (Farese 1990, Fonteles et al. 1996, Gaulton 1994, Larner 1987, Lazar et al. 1994, Lisanti et al. 1989, Mato 1989, Low and Saltiel 1988, Lawrence et al. 1986, Müller 2002a, Romero 1990, Shaskin et al. 1997, Saltiel et al. 1988).

K.6.3.6.4

Glycosyl-Phosphatidylinositol-Specific Phospholipase (GPI-PL) and Insulin-Like Signaling

GENERAL CONSIDERATIONS

Insulin-responsive mammalian cells and tissues express a number of GPI-proteins, the majority of them with their GPI anchor embedded in the outer leaflet of the plasma membranes as well as some lipases, the (G)PI-PLC/D, which may control down-regulation of GPI-protein cell surface expression and simultaneously up-regulation of the soluble protein moiety released into circulation (Romero et al. 1988, Low 1989 and 1990, Thomas 1990, Romero and Larner 1993, Saltiel 1990, Saltiel et al. 1986, Müller and Bandlow 1991, Varela-Nieto et al. 1996, Nosjean et al. 1997, Jones and Varela-Nieto 1999, Müller et al. 2005). In fact, soluble forms of GPI-proteins have been detected in human plasma, such as 5'-nucleotidase, Thy-

PURPOSE AND RATIONALE

Recently, a synthetic inositol derivative, GPI-2350, has been described which inhibits the well-characterized bacterial, trypanosomal and serum (G)PI-PLC/D with high potency and selectivity (Müller et al. 2005). Using GPI-2350, which almost completely down-regulates the insulin- and glimepiride-inducible GPI-PLC in intact rat adipocytes, a major role for this enzyme in metabolic insulin signalling and action in

these cells could be excluded. In contrast, activation of the GPI-PLC turned out to be indispensable for the insulin-mimetic effects of glimepiride *via* the recently described IR-independent cross-talk from lipid rafts to the IRS-1 protein (see above). Based on the criteria for lipid rafts, certain GPI-anchored, acylated and transmembrane signalling proteins have been found to be enriched in rafts *vs* non-raft areas of the plasma membranes. Furthermore, rafts are not homogenous in composition and structure since subspecies of higher (hcRafts) and lower cholesterol (lcRafts) content can be distinguished from one another on the basis of their lower and higher buoyant density, respectively (Müller 2005, Müller et al. 2005). Strikingly, the stimulus-induced redistribution of certain GPI-anchored as well as acylated signalling proteins from hcRafts to lcRafts (Müller et al. 2001a, b) is blocked by GPI-2350. This finding hints to a role of the mammalian GPI-PLC in the control of the localization and activation of signalling proteins within rafts during signal transduction across the plasma membrane of adipocytes. The following assays enable the analysis of the role of the GPI-PL for insulin-like signaling and metabolic action in insulin target cells as well as of the effects of compounds/drug candidates on the activity of the GPI-PL. The GPI-PL may turn out as promising target for future anti-diabetic drugs (Müller and Frick 1999, Müller 2002b, Müller and Welte 2002).

K.6.3.6.4.1

Activity Assay for GPI-PL

PROCEDURE

Adipocyte GPI-PLC

Adipocyte GPI-PLC (5–25 µg PM or hc/lcRafts) is assayed as the conversion of GPI-protein, acetylcholinesterase (AChE) from its amphiphilic version (containing the intact GPI anchor) into its hydrophilic version (containing the lipolytically cleaved GPI anchor) are assayed according to a protocol adapted from Müller and coworkers (1994b) by incubation with 10 µl of bovine erythrocyte AChE solution (12 U/ml in 10 mM Tris/HCl, pH 7.4, 144 mM NaCl, 0.1% TX-100) in 100 µl of 20 mM HEPES/KOH (pH 7.8), 144 mM NaCl, 0.1% TX-100, 0.2 mM MgCl₂ for 1 h at 25°C and subsequent termination by adding 5 µl of glacial acetic acid and then 0.4 ml of 10 mM Tris/HCl (pH 7.4), 144 mM NaCl. Each reaction mixture is subjected to TX-114 partitioning (see below). The GPI-PLC activity is calculated as the amphiphilic-to-hydrophilic conversion of the GPI-protein substrates from the ratio of the activities of hydrophilic 5'-Nuc or AChE measured in the TX-114-depleted phase and

the total activity measured before partitioning and corrected for the non-enzymatic background in the TX-114-depleted phase (accounting for 10–20% of the total activity) as revealed by blank incubations lacking (G)PI-PLC.

TX-114 partitioning

Pelleted hc/lcRafts (10–50 µg protein) or GPI-PL reaction mixtures (0.5 ml) are separated into amphiphilic and hydrophilic proteins using partitioning between TX-114-enriched and depleted phases according to Bordier (1981) and Pryde and Phillips (1986) by suspending in 1 ml or mixing with 0.5 ml, respectively, of ice-cold 25 mM Tris/HCl (pH 7.4), 144 mM NaCl containing 1 or 2% TX-114. After incubation for 1 h on ice, the mixture is layered onto a cushion of 0.4 ml of 0.25 M sucrose and 25 mM Tris/HCl (pH 7.4) on ice. Phase separation is induced by warming up to 37°C and subsequent centrifugation (10,000 × g, 1 min). After re-extraction of the lower TX-114-enriched phase, aliquots of the pooled upper TX-114-depleted phase are measured for AChE activity or precipitated (15% polyethylene glycol 4000) for SDS-PAGE analysis.

EVALUATION

Concentration-response curves are established using various concentrations of standard (human insulin) and the test compounds, e. g. sulfonylureas, allowing calculation of EC₅₀ values and potency ratios.

K.6.3.6.4.2

GPI-PL-Dependent Translocation of GPI Proteins Within Lipid Rafts

PROCEDURE

Photoaffinity Labeling of the GPI Protein, Gce1

Solubilized plasma membranes and rafts (5–10 µg protein) are incubated (30 min, 4°C) with 50 µCi 8-N₃-[³²P]cAMP (0.5 nmol) in 50 µl of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM isobutylmethylxanthine, 1 mM DTT, 1 mM AMP, and protease inhibitors in the wells of microtiter plates (96-formate) and then irradiated with UV light (254 nm, 8000 µW/cm²) at a distance of 0.5 cm for 1 min (Müller et al. 1993, 1994a, b). Subsequently, the photoaffinity labeling reaction is quenched by addition of 100 µl of the same buffer containing 10 mM cAMP. Gce1 is precipitated (5% trichloroacetic acid, 1 h on ice, 10,000 × g, 15 min) and solubilized in sample buffer for SDS-PAGE. Protein concentration is determined using the BCA protein determination kit from Pierce. Autoradiographs and direct photoimages

are processed and quantified by computer-assisted video densitometry (e.g. Storm 860 PhosphorImager system, Molecular Dynamics). The recovery in the amounts of immunoprecipitated protein has to be corrected (data on fold- or % stimulation) for the amount of protein actually applied onto the gel as revealed by homologous immunoblotting.

Preparation of lc/hcRafts

Pre-treated and subsequently washed adipocytes are homogenized in lysis buffer (25 mM MES, pH 6.0, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.25 M sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM glycerol-3-phosphate, 1 mM sodium orthovanadate and protease inhibitors) using a motor-driven Teflon-in-glass homogenizer (10 strokes). Plasma membranes are obtained by differential centrifugation of the defatted postnuclear infranant (= cytosolic fraction) and then purified by two sequential centrifugation steps through sucrose and Percoll cushions as described above and finally suspended in 25 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA at 2 mg protein/ml. hc/lcRafts are obtained by the detergent method and discontinuous sucrose gradient centrifugation as reported above. The light-scattering opalescent bands at the 0.5–0.65 M (fractions 3 + 4) and 0.8–0.9 M (fractions 5 + 6) sucrose interfaces are collected as hcRafts and lcRafts, respectively, with density measurement using the refractive index. hc/lcRafts are collected by centrifugation (50,000 × g, 30 min, 4°C) after three-fold dilution of the pooled gradient fractions with 25 mM MES (pH 6.5), 1% TX-100, 150 mM NaCl, and then characterized (immunoblotting, enzyme assays) by enrichment/deprivation of relevant marker proteins as described above or subjected to TX-114 partitioning (see K.6.3.6.4.1). For immunoprecipitation of caveolin, rafts are solubilized (1 h, 4°C) in 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% TX-100, 60 mM β-octylglucoside, 0.3% deoxycholate, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 1 μM microcystin and protease inhibitors and centrifuged (50,000 × g, 30 min). For direct immunoblotting, rafts are solubilized in two-fold Laemmli sample buffer and centrifuged (10,000 × g, 5 min). The supernatants are used.

EVALUATION

The following working hypothesis for signal generation in and transduction across the PM of rat adipocytes on the basis of the redistribution of lipid-modified signalling proteins, such as GPI-proteins and

acylated tyrosine kinases, within rafts has been deduced from the present experimental findings (Müller 2005). (1) Amphiphilic GPI-proteins with intact GPI anchor as well as hydrophilic GPI-proteins with lipolytically cleaved GPI anchor preferentially accumulate at hcRafts versus lcRafts. (2) Only uncleaved GPI-proteins redistribute from hcRafts to lcRafts in response to glimepiride and insulin. Apparently, the GPI anchor functions as a signal for the recruitment to rafts *versus* non-raft areas, but does not determine the targeting to lcRafts or hcRafts. The current working model implicates that the PIG core structure of the GPI anchor functions as the only hcRafts-targeting signal (*via* recognition through the receptor protein, p115) for a subset of GPI-proteins which are amenable to stimulus-induced redistribution to lcRafts. The present findings strongly suggest the expression of three distinct subspecies of rafts in the plasma membranes of rat adipocytes: (i) TX-100- and Lubrol-WX-insoluble hcRafts, (ii) TX-100-soluble and Lubrol-WX-insoluble lcRafts in the basal state and (iii) TX-100- and Lubrol-WX-insoluble lcRafts upon glimepiride challenge. This is more compatible with the model of heterogenous rafts rather than that of layered rafts or homogenous rafts with selective lipid extraction as has been recently proposed for their heterogenous structure as well as their biogenesis and maintenance (Macdonald and Pike 2005). Accordingly, layered rafts are constituted of rings ranging from a liquid-ordered cholesterol- and glycosphingolipid-enriched core through less ordered regions into the liquid-disordered non-raft areas of the residual PM, the core of which is left as hcRafts after TX-100 solubilization, whereas core and immediately surrounding areas are left unsolubilized by Lubrol-WX. Homogenous rafts are liquid-ordered domains of a uniform mixture of the protein and lipid constituents are surrounded by the liquid-disordered phase of the plasma membranes which are selectively solubilized by TX-100 and Lubrol-WX (Macdonald and Pike 2005). Both models can hardly explain the existence of TX-100-insoluble lcRafts or their generation from hcRafts in response to glimepiride. In contrast, heterogenous rafts of different lipid and protein composition could coexist as separate structural entities in the membrane and be differentially solubilized by TX-100 and Lubrol-WX. It is conceivable that the glimepiride-induced translocation of certain GPI-anchored and acylated signalling proteins from distinct hcRafts accompanied by the limited loss of cholesterol results in the *de novo* generation of lcRafts of increased buoyant density retaining TX-100 insol-

ubility to a partial degree. Alternatively, the translocated raft protein components may be recruited directly from discrete hcRafts into pre-existing Lubrol-WX-insoluble (TX-100-soluble) lcRafts thereby rendering them partially insoluble in TX-100.

In conclusion, the adipocyte GPI-PL has now been recognized as a critical component specifically operating in the insulin-like signalling cascade triggered by glimepiride rather than in insulin signalling. Furthermore, insulin-like signalling by glimepiride is not significantly impaired in the insulin-resistant state as reported previously for rat adipocytes *in vitro* (Müller and Wied 1993). Therefore, the adipocyte GPI-PLC might represent a useful target for the therapy of type II diabetes mellitus.

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- of the gene expression for proteins/enzymes which directly regulate carbohydrate and lipid metabolism (e. g. GLUT4, PEPCK). Furthermore, changes in the intermediary metabolism provoked by insulin may secondarily lead to alterations in the gene/protein expression of other signalling/metabolic pathways. Consequently, it is important for the identification as well as characterization of compounds/drug candidates with insulin-like activity to analyze their effect on the expression of gene and proteins. For practical reasons, this analysis can be restricted to those genes/proteins which according to the present knowledge are relevant for mode of action of the compound/drug candidate. However, undoubtedly whole-genome or proteome searches for changes in the expression levels have important advantages, in particular due to its unbiased nature. However, they require considerable experimental expenditure and time on basis of conventional technology. This may change completely with the successful introduction of protein chips and DNA microarrays for the study of protein and gene expression and further increase its attractiveness for routine application in characterization of novel compounds/drug candidates.

K.6.4.1 Protein Chips

GENERAL CONSIDERATIONS

Currently, mapping the proteome and understanding the roles of these proteins are still in the very early stages, but scientists are hopeful that individual proteins, as well as constellations or “fingerprints” made up of multiple proteins (even if those proteins’ functions are unknown), will soon be used regularly for the characterization of compounds/drug candidates in drug discovery programmes, in addition to application in the characterization of the probands for clinical trials regarding both desired and undesired effects of drug candidate envisioned. Indeed, the promise of these tests is so significant that many companies are already using such markers to select the most appropriate patients for clinical trials and to choose better drug candidates. Meanwhile, pharmaceutical and biotechnology companies have become acutely aware of the value of biomarkers for identifying those patients who may disproportionately benefit from a drug and those at highest risk of experiencing serious adverse effects. Biomarkers are regarded as a vital tool in streamlining drug discovery and development and accelerating approvals.

K.6.4

Assays for Insulin and Insulin-Like Regulation of Gene and Protein Expression

Part of the blood glucose-lowering activity of insulin is based on massive and specific up- or downregulation

Proteins are the workhorses of the cell. Although genes represent the blueprint of life, proteins are the molecules that actually carry out the gene's instructions. In addition, because proteins are not confined to the cell that created them (as is mRNA) but float throughout the bloodstream, they are easier to find and measure. During the pre- and post-genome-sequencing euphoria of the late 1990s and early 2000, many researchers declared that the proteome was the next frontier.

K.6.4.1.1

Challenges for Development

A major obstacle in protein chip development is the fact that proteins greatly outnumber genes. A single gene can code for multiple proteins, and each protein can undergo modifications that substantially change its activity. As a result, potentially several hundred thousand proteins can be required for the estimated 30,000 genes to do their work in the human body. This scenario helps explain the complexity of the human body despite the fact that it has relatively few genes compared with lower organisms. It also makes it much harder for scientists to completely map the proteome. In addition, in contrast to the strandlike construction of mRNA and DNA, proteins have extremely complex structures. Within each protein's convoluted architecture there are certain "active" sites that make the most difference in terms of the protein's activity (It is in these sites that other molecules, including drugs, exert their activity). When they are placed on a substrate, such as a chip, proteins may unfold, becoming inactive and useless for study.

In terms of the technology, the traditional method of using 2-D gels for measuring the protein expression in a clinical or experimental sample is tedious and cumbersome. Initially, there was great interest in finding ways to industrialize this approach by using mass spectrometry and automated systems. With the arrival of DNA microarrays and their success in the RNA measurement field, the dawn of "proteome-wide chips" seemed imminent. But the technological hurdles facing the field have not diminished substantially and throughput in proteomics is still far behind what is achievable in areas such as gene sequencing and gene expression analysis.

As a result of the aforementioned challenges, many groups have taken a tip from the surging interest in gene-expression-profiling patterns. To develop such "signatures" of disease, researchers realized that they need only capture several proteins accurately and re-

producibly. The protein marker signature does not need to be understandable. It just needs to be robust. Thus, interest in proteomics has now switched from being able to accurately quantify and identify all of the proteins in a particular sample to being able to distinguish "fingerprints" that are sufficient to separate one sample (e. g. progressive disease) from another (e. g. mild disease).

K.6.4.1.2

Technologies

Protein chips are mainly used to either identify protein-protein interactions or to find proteins in a sample. The former type of chip is primarily a drug discovery and development tool, while the latter type can be used either in drug discovery and development or in diagnostic development. The type of molecule used to capture the proteins is the fundamental difference between chips for research purposes, which can be used to find biomarkers, and those chips that will be used to diagnose disease or predict outcomes. When searching for protein biomarker signatures, one must be sure they are examining a wide range of proteins and accurately measuring the relative levels of all of these proteins. Once the markers have been identified, one can then concentrate on a particular subset of proteins and find the ideal binding-molecules for use in a diagnostic system. Therefore, the key intermediate step is to locate the important marker proteins among the several hundred thousand proteins present in the body. A variety of molecules can be used to bind proteins, but antibodies are most often used. Primary antibodies are used to capture the protein (antigen) which is recognized by secondary antibodies (not sharing the antigenic determinant with the primary antibody but often labeled with biotin), and some type of read-out (e. g. Cy5-labeled streptavidin) signals presence of the protein (antigen). To build such arrays, each antibody placed on the chip (often in the wells of a microtiter plate) must be generated and then thoroughly tested for its cross-reactivity and binding affinity. Because proteins can share domains, or certain common structures, cross-reactivity can cause serious problems. Determining that a protein is cross-reactive requires extensive screening against other proteins. In addition, it is very difficult to get antibodies that bind tightly to a particular protein. Haab and coworkers (2001) showed that as few as 20% of antibodies out of hundreds could provide specific and accurate measurements of matching proteins on a chip. Antibodies can be exquisitely specific, however, and researchers are only now begin-

ning to learn some general rules on how to design good antibodies for proteins microarrays. A certain number of antibodies are already very well characterized. The pharmaceutical industry has been heavily reliant on these identified antibodies. The technology for making protein chips has also advanced, largely through the development of new methods to capture the proteins without degrading them.

New approaches to protein microarrays are also emerging. For example, mass spectrometry has recently been demonstrated to be useful for selection of certain proteins from a mixture and then to “soft land” these proteins onto a microarray while retaining their shape and activity to a very high degree. Meanwhile, a bench-top system of arrays of silicon-nitride microcantilevers with customizable surfaces has been introduced to detect interactions between proteins, antigens and DNA. This system does not require labelling because the microcantilevers bend due to the interaction between two molecules. Furthermore, a digital proteome chip has been developed for a comprehensive protein expression analysis. The technology is predicted of being unusually rapid (a single sample can be processed in one hour or less) and has unprecedented sensitivity, reproducibility, and accuracy. Very recently, isothermal rolling-circle amplification technology (RCAT) has been linked to antibodies as a means of signal amplification on traditional sandwich immunoassay-based protein chips. This detection method may be particularly useful because it remains localized and can detect protein analytes with zeptomole sensitivity and across a very broad dynamic range. Protein microarrays for interaction studies and other functional genomics studies will be most useful for drug discovery and development. By using a parallel procedure for protein production and purification technology it is assumed to generate and purify thousands of different proteins for the generation of the most diverse “portfolio of functional protein content”. A comparative evaluation of selected suppliers of protein chip technology including their applications reported so far has been provided by Branca (2004).

K.6.4.2

DNA-Microarrays

PURPOSE AND RATIONALE

Changes and improvement in microarray technology have enhanced the ability to analyze transcription-factor-binding events genome-wide. Early versions of microarrays used for location analysis were manufactured by amplifying the promoter regions of genes

from genomic DNA using PCR, and spotting the amplicons on glass slides. In addition to being reasonable accessible and affordable, PCR-generated probes could cover the entire proximal promoter region of a gene with a single probe. But these arrays had limitations. Double-stranded PCR products are less effective hybridization probes than single-stranded oligomers. PCR-generated probes are more susceptible to variations in quality due to the processing required to generate them and with only one probe reporting for each promoter, there is a relatively high rate of error associated with bad probes. Finally, the binding may occur anywhere within the probe sequence, which may be as long as 1 or 2 kB.

PROCEDURE

Emerging microarray applications such as aCGH (array-based comparative genomic hybridization), SNP (single nucleotide polymorphism) analysis, location analysis (ChIP, on-chip-chromatin immunoprecipitation on a microarray), methylation analysis, splice variant analysis and microRNA studies are all growing faster even than the already popular microarray application of gene expression. Inkjet-based array manufacturing processes provide the flexibility, long probe lengths and density required by these applications. The use of ChIP and microarray technology has proved a powerful combination for analyzing transcriptional regulation (for a review see Lee and Volkert 2006). One of the primary advantages of ChIP is that protein-DNA interactions are first fixed by the addition of a cross-linking agent to living cells, thus allowing for the identification of dynamic *in vivo* interactions between transcription factors and the genome. However, this approach has been relatively limited for exploring protein-DNA interactions across the genome as detection of the enrichment on this scale can be difficult. Enrichment is often detected by use of PCR-based assays, which are easy to perform and highly informative, but detection is limited because it is difficult to perform these assays in a sufficiently high-throughput format for genome analysis. Alternative approaches, such as cloning and sequencing of enriched DNA or direct sequencing of enriched DNA, have also been used, but are relatively labour-intensive and depend on sequencing large numbers of fragments to approach complete genome coverage. Using microarrays for the identification of enriched DNA regions has circumvented some of the difficulties in detecting enrichment genome-wide. Immuno-enriched DNA can be labeled, hybridized to arrays and compared to control DNA that has been hybridized at the same time. As arrays can be

designed to probe all known promoter regions (Boyer et al. 2005) or even the entire genome (Kim et al. 2005), this ensures complete coverage of the genome in a rapid and highly parallel fashion.

The current versions of arrays used for location analysis take advantage of newer printing technology. Features are now commonly composed of 60-mer oligonucleotides rather than long PCR amplicons. Several studies have shown that oligonucleotide probes are more sensitive than PCR probes in microarray analysis (Carter et al. 2003, Hughes et al. 2001, Li et al. 2002). Furthermore, it has been shown that results using longer oligonucleotides are more sensitive and reproducible than shorter ones. The 5070-base range seems to represent the “sweet spot” where hybridization efficiency, specificity and sensitivity are co-optimized. These oligonucleotide arrays are manufactured by commercial vendors, which translates to an increase in both the quality and consistency of the arrays. For instance, there are currently three array designs for the study of transcription factor binding in the human genome available (for a review see Lee and Volkert 2006).

EVALUATION

Future studies will focus on expanding the transcriptional regulatory network for metabolic genes. It will be interesting to identify the targets of transcription factors that are themselves targets of metabolic transcription factors, thus expanding the transcriptional regulatory network responsible for the genetic programme underlying the coordination of glucose and lipid metabolism. Transcription factors are responsible for recruiting additional components of the transcription apparatus, most notably chromatin-remodelling and –modifying complexes that affect transcription by regulating higher-order DNA structure. Consequently, one area of interest is identifying the targets of these various remodelling and modifying complexes and linking the network of transcription factors with the network of chromatin modifiers in liver, muscle, adipose and pancreatic β -cells.

K.6.4.3 siRNA

During the past decade a panel of methods has been developed for the validation of drug targets at the cellular level encompassing constitutive or regulated overexpression of the gene envisioned or its downregulation (antisense RNA, mouse embryonic fibroblasts from knockout mice and RNAi). RNAi displays a number

of advantages regarding the expenditure in equipment, costs and time required. Moreover, the incomplete efficacy of RNAi in downregulating gene expression (usually by up to 80%) more closely reflects the pharmacological inhibition of transcription by small molecule compounds. In addition, there are considerable efforts to use RNAi as future therapeutics albeit many challenges have to be overcome.

RNA interference (RNAi) is an ancient mechanism of gene regulation, found in eukaryotes as diverse as yeast and mammals, and probably plays a central role in controlling gene expression in all eukaryotes (Tijsterman et al. 2002). Using small interfering RNA (siRNA) molecules, RNAi can selectively silence essentially any gene in the genome. Once in a cell, a short double-stranded RNA (dsRNA) molecule is cleaved by an RNase III, called “Dicer”, into 21- to 23-nucleotide guide RNA duplexes with two-nucleotide 3'-overhangs, called siRNAs that become bound to the RNA-induced silencing complex (RISC). Within the RISC, one of the two strands of the siRNA is chosen as the antisense strand *via* cleavage of the passenger strand (Gregory et al. 2005, Rand et al. 2005), so that they can target complementary sequences in messenger RNAs involved in a disease. An RISC-associated “slicer” RNase belonging to the Argonaute2 (Ago2) family then cleaves the target in the middle of the homology, which is followed by further degradation mediated by an exosome complex (Liu et al. 2004, Rand et al. 2004) thereby interrupting the synthesis of the disease-causing protein. The RISC complex is naturally stable within the cell, enabling siRNA to cut multiple mRNA molecules consecutively and, therefore, suppressing protein synthesis in a potent and targeted way.

K.6.4.3.1 Design

The bulk of pharmaceutical applications of RNAi uses siRNA or its precursor. A completely foolproof algorithm of siRNA design for every target may remain an unattainable dream due to the inherent difficulty of predicting the various parameters that affect siRNA function (Heale et al. 2005, Amarguoui et al. 2004, Gong and Ferrell 2004). However, some significant factors have been recognized. First, a successful application of RNAi demands that it be as specific to the target RNA as possible. Kim and coworkers (2004) demonstrated that 27-bp siRNAs are 10- to 100-times more potent and specific *in vivo* than their 21-bp counterparts. Second, Schwarz and coworkers (2003) found

that the formation of RISC is an asymmetric process in that only one strand of the siRNA is incorporated, not both. Third, it is now clear that the local secondary structure of the target RNA has dramatic influence on the accessibility for the siRNA (Vickers et al. 2003, Kretschmer-Kazemi and Sczakiel 2003, Luo and Chang 2004). Although siRNA designs have been proposed to incorporate this parameter (Heale et al. 2005, Amarzguioui and Prydz 2004, Gong and Ferrel 2004), the empirical nature of RNA structure prediction has left room for uncertainty. Fourth, a number of studies have reported various degrees of positive and negative regulation of genes besides the intended target leading to “off-target” effects that are not easily explained by a fortuitous sequence homology of the siRNA to the off-target mRNAs (Sledz et al. 2003, Jackson et al. 2003, Bridge et al. 2003). In conclusion, one should follow all the guidelines suggested above but remember that the ultimate test is experimental.

K.6.4.3.2

Source

It is to be realized that RNAi-based target validation or therapy exploits the physiological machinery constitutively present in the normal cell and that dsRNA is the only entity needed to activate it. In other words, therapeutic RNAi is triggered by the simple introduction of target-specific siRNA. This is commonly achieved by one of the following methods. The 21-mer siRNA with the 3'-dinucleotide overhangs can be chemically synthesized and introduced into the cell. Alternately, relatively long sense and antisense strands are transcribed *in vitro* from recombinant DNA templates, annealed to produce the dsRNA, which is digested with Dicer to generate siRNA that is then transfected into the cells. Lastly, the dsRNA can be generated *in vivo* by transfection of the corresponding DNA clone, which is then processed by the intracellular Dicer to generate the siRNA.

Synthetic siRNA

The chemistry of RNA synthesis has significantly improved over the years with an additional boost from the recent demands of the RNAi market. Short RNAs containing a variety of modifications are now commercially available (Dorsett and Tuschl 2004, Manoharan 2004). Modifications are explored for a variety of goals, such as improved RNAi activity and lower IC₅₀, enhanced stability, particularly against nucleases, and better pharmacokinetics and organ targeting. Effect of terminal and internal modifications of the siRNA on

the silencing activity has been tested, and the consensus is summarized here. Additions at the 5'-end of the sense strand had little or no effect (Chiu and Rana 2003). A 5'-phosphate group on the antisense strand is essential for the incorporation into RISC. However, additions to the phosphate group are generally tolerated (Nykanen et al. 2001, Martinez et al. 2002). Tolerance at the 3'-end of the antisense depends on the derivative. While biotin, ddC, aminopropyl group and puromycin do not inhibit siRNA activity, 2-hydroxyethylphosphate, ethylene thymidine, and some fluorescent derivatives do (Schwarz et al. 2002). Of all the internal modifications, substitutions of the 2'-OH of ribose are the best studied and include -H, -OMe, -F and -NH₂. In general, all 2'-substitutions in the 3'-overhangs are tolerated (Elbashir et al. 2001, Amarzguioui et al. 2003). siRNAs with internal 2'-F are RNase-resistant and may perform better than unmodified siRNA in cell culture (Capodici et al. 2002), but not necessarily so in animals (Layzer et al. 2004). Internally, 2'-O-methylated siRNAs also exhibit significantly greater stability in serum with sustained silencing activity, and siRNAs with alternate 2'-OMe and 2'-F substitutions have similar advantages as well. For reasons that are unclear, 2'-NH₂ substitutions in either sense or antisense strand cause significant loss of silencing activity (Parrish et al. 2000). Replacement of the phosphodiester (P=O) backbone with phosphorothioates (P=S) or boranophosphonates (P=B) also offers nuclease resistance, but excessive substitution leads to loss of activity and/or increased toxicity (Manoharan et al. 2004, Amarzguioui et al. 2003). Lastly, siRNA containing synthetic RNA-like nucleotide analog, known as “locked nucleic acid” (LNA), exhibits greatly improved biostability and enhanced inhibition of certain RNA targets (Braasch et al. 2003).

siRNA Transcribed In Vitro

Exogenous siRNA is also produced by the transcription of both strands of recombinant DNA templates, usually from a bacteriophage promoter, such as T7 or SP6 (Dudek and Picard 2004). As the resultant transcripts have 5'-triphosphates that may activate the undesirable IFN pathway, as mentioned before (Kim et al. 2005), the dsRNA product is either treated with phosphatase or trimmed with Dicer (Myers et al. 2003) *in vitro* before transfection.

Recombinant siRNA

The other common technique is to transfect target cells with DNA clones expressing shRNA, also known as DNA-directed RNAi or ddRNAi (Smith et al. 2000).

The corresponding complementary DNA (cDNA) is cloned between two converging promoters, generating dsRNA in the recipient cells that is cleaved by cellular Dicer into siRNA. In an alternate strategy, a DNA segment containing a hyphenated inverted repeat produces an shRNA of 19 bp or longer upon transcription. The shRNA is processed essentially by the miRNA pathway using Droscha and Dicer as described previously to eventually generate the siRNA. Both strategies employ RNA polymerase III promoters, such as human and mouse U6, human H1, and human 7SK promoters. The commonly used vectors are of adenoviral, lentiviral, or retroviral origin. Recombinant expression opens the avenue for inducible and/or tissue-specific expression of the siRNA (Wiznerowicz et al. 2003, Matsukura et al. 2003), which is not possible with synthetic siRNA. It also allows more sustained siRNA production over longer periods, critical in therapy for chronic diseases (see below). The potential hazards include viral infection, adverse immune reactions, and disruption or dysregulation of important genes via chromosomal integration of the viral genome (Bartosch and Cosset 2004). Furthermore, the parameters of shRNA design (such as the optimal length of the stem and the loop regions) are still ill defined and need further experimentation.

K.6.4.3.3

Delivery

There are two strategies for delivering siRNAs *in vivo*. One is to stably express siRNA precursors, such as short hairpin RNAs (shRNAs) from viral vector using gene therapy. The other is to deliver synthetic siRNAs by complexing or covalently linking the duplex RNA with lipids and/or delivery proteins. To solve the problem of cell penetration, most researchers have either complexed the RNA with a lipid or modified the RNA's phosphate backbone to minimize its charge. Despite the questions and unresolved problems, several companies are moving ahead to develop RNAi therapy for many diseases, including diabetes. The standard lipid reagents that were originally developed for DNA transfection, such as Oligofectamine (Invitrogen), continue to be the reagents of choice for cell culture studies (Spagnou et al. 2004). Recent formulations claim to be specifically improved for RNA delivery and can be used in the presence of serum in cell culture and in live animals. Current options for the synthesis and design has been reviewed by Baric (2005).

K.6.4.3.4

Use for Metabolic Diseases

Numerous studies using highly sensitive microarray analyses have demonstrated that siRNAs can have off-target effects by silencing genes relevant for the regulation of carbohydrate and lipid metabolism in adipose, liver, muscle and pancreatic β -cells. This makes siRNA to an ideal tool for the cellular validation of molecular targets (e. g. enzymes, transcription factors) for the therapy of metabolic disorders, such as diabetes and obesity, as has been demonstrated elegantly by Zhou and coworkers (2004). In addition, RNAi-based therapeutics have potentially significant advantages over traditional approaches of treating diseases, including broad applicability, therapeutic precision, and selectivity avoiding side effects. This widespread applicability, coupled to relative ease of synthesis and low cost of production make siRNAs an attractive new class of small-molecule drugs. RNAi-based drugs are designed to destroy the target RNA and therefore stop the associated undesirable protein production required for disease progression. The putative therapeutic potential for the use of siRNA, in general, and the cure of metabolic diseases, particular, has been reviewed by Dallas and Vlassov (2006) and Rondinone (2006).

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K.6.4.4**Effect on Peroxisome Proliferator-Activated Receptor****GENERAL CONSIDERATIONS**

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of

the nuclear receptor superfamily (Issemann and Green 1990, Dreyer et al. 1992). They regulate glucose, lipid, and cholesterol metabolism in response to fatty acids and their derivatives, eicosanoids, and drugs used in the treatment of hyperlipidemia and diabetes. The PPAR subfamily contains three members known as PPAR α , PPAR β/δ , and PPAR γ . Each PPAR subtype shows a distinct tissue distribution (Keller and Wahli 1993, Green 1995, Devchand et al. 1996, Lemberger et al. 1996, Schoonjans et al. 1996a, b, c, 1997, Willson et al. 2000, Walczak and Tontomoz 2002) and ligand preference.

PPAR α is expressed predominantly in metabolically active tissues, including liver, kidney, skeletal muscle, and brown fat (Sher et al. 1993, Mukherjee et al. 1994), and its ligands include fatty acids, hypolipidemic drugs, eicosanoids and xenobiotics (Forman et al. 1997, Kliewer et al. 1997, Forman et al. 1995, Devchand et al. 1996, Lehmann 1995).

PPAR γ is highly expressed in adipocytes (Elbrecht et al. 1996, Mukherjee et al. 1997), is involved in control of lipid storage, and plays a critical role in the regulation of adipocyte gene expression and the induction of adipogenesis and adipocyte differentiation (Tortonoz et al. 1994 and 1995, Brun et al. 1996, Wu 1998, Lowell 1999). Thiazolidinedione derivatives which are antidiabetic agents are potent and selective activators of PPAR γ (Young et al. 1997, Henke et al. 1998, Murakami et al. 1998, Reginato et al. 1998, Ribon et al. 1998, Vázquez et al. 2002). The meanwhile widely used anti-diabetic drugs, pioglitazone and rosiglitazone, preferentially bind to the γ -isoform and modulate the expression of a number of genes in predominantly adipose tissue, but presumably also in muscle and liver tissues, ultimately leading to substantial increase in insulin sensitivity (Lehmann et al. 1995). Berger and coworkers (1996) found a correlation of antidiabetic actions of thiazolidinediones in *db/db* mice with the conformational change in PPAR γ . Su and coworkers (1999) described the use of a PPAR γ -specific monoclonal antibody to demonstrate thiazolidinediones induced PPAR γ receptor expression *in vitro*.

Murphy and Holder (2000) suggested a therapeutic potential of PPAR γ agonists in the treatment of inflammatory diseases and certain cancers. Stepan and coworkers (2001) and Berger (2001) showed that adipocytes secrete a signaling molecule which they called resistin (for resistance to insulin). Circulating resistin levels in mice are decreased by thiazolidinediones and are increased in diet-induced and genetic forms of obesity.

Although the function of PPAR δ , which is expressed ubiquitously, is less well known, this nuclear receptor may be involved in the regulation of cholesterol and lipid metabolism (Oliver et al. 2001, Shi et al. 2001). Fatty acids and cyclooxygenase 2-derived prostacyclin (PGI₂) are natural ligands for PPAR δ (Gupta et al. 2000). Several highly selective and potent synthetic ligands (e. g., L-165041, GW501516) activate PPAR δ at nanomolar concentrations (Oliver et al. 2001, Glinghammar et al. 2003).

All subtypes share a common structural organization with a highly conserved N-terminal DNA binding domain (DBD) and a moderately conserved C-terminal ligand-binding domain (LBD) that contains a carboxy-terminal activation function motif (AF2). All of the PPAR subfamily members heterodimerize with the receptor for 9-*cis* retinoic acid (RXR) and bind to target gene peroxisome proliferator elements (PPREs), a direct repeat of the sequence AGGTCA separated by one nucleotide (DR-1) (Dreyer et al. 1992, Kliewer et al. 1992).

Unliganded nuclear receptors, such as retinoic acid receptors (RAR α) and T3R α , are able to repress basal transcription through recruitment of corepressors (Chen and Evans 1995). Among the three PPARs, PPAR δ distinguishes itself by displaying remarkably potent transcriptional repression activity. In contrast, unliganded PPAR α and PPAR γ do not repress PPRE-mediated transcription, presumably due to their inability to bind nuclear receptor corepressors SMRT and NCoR on the PPRE DNA element (Shi et al. 2001). Agonist binding to nuclear receptor LBD provokes a conformational change of the AF-2 motif that produces a suitable binding surface for recruitment of coactivators (Xu et al. 1999).

K.6.4.4.1

Recombinant Cell Lines

PURPOSE AND RATIONALE

Because of involvement of PPARs in many critical physiological and pathological functions, in particular with regard to metabolic diseases, the identification of high-affinity ligands would create useful tools for studying the PPAR role in the normal physiological and disease states as well as for drug discovery, in particular for the therapy of metabolic diseases, such as type II diabetes. To characterize PPAR specificity of synthetic ligands, stable HeLa-derived reporter cell lines in which PPAR α , PPAR β , and PPAR γ agonists induce luciferase activity have been constructed. These cell lines stably express a chimeric protein con-

taining the yeast transactivator GAL4 DBD fused to LBD regions of PPARs (GAL4-PPARs) and contain a luciferase reporter gene driven by a pentamer of the GAL4 recognition sequence in front of the β -globin promoter. The GAL4-PPAR chimeric receptors has been chosen because this assay format eliminates background activity from endogenous receptors and allowed quantitation of relative activity across the three PPAR subtypes with the same reporter gene. Additional assays for the characterization of PPAR-modulating compounds and drug candidates are given in K.3.5.

K.6.4.4.2

Lipogenesis Assay

The lipogenic activity of PPAR γ agonists can be assessed by determining the uptake of radioactive glucose uptake and its incorporation into the total lipids of cells (see K.6.1.2.1). The amount of glucose incorporation into lipids in these cells provides a measure of cell differentiation, since preadipocytes are less efficient in *de novo* lipogenesis compared to mature adipocytes on basis of their different expression levels of lipogenic genes, such as GLUT4 and GPAT. Moreover, the analysis of the expression of adipocyte marker genes (aP2, LPL, leptin) and of the accumulation of TAG in the cytoplasm confirmed by oil red O staining are other methods to evaluate the lipogenic activity of PPAR γ agonists and thereby indirectly their binding affinity to PPAR γ .

K.6.4.4.3

Protease Digestion Assay

PROCEDURE

The protease digestion assay can be performed according to the method of Allan and coworkers (1992) with minor modifications. The plasmid pSG5-hPPAR γ 1 is used to synthesize ^{35}S -radiolabeled PPAR γ 1 in a coupled transcription/translation system. The transcription/translation reactions are subsequently divided into portions of 22.5- μl volume. 2.5 μl PBS with or without a thiazolidinethione are added. The mixtures are incubated for 20 min at 25°C, separated into 4.5- μl aliquots, and 0.5 μl distilled water or distilled water-solubilized trypsin are added. The protein digestions are allowed to proceed for 10 min at 25°C, then terminated by the addition of 20 μl denaturing gel loading buffer and boiling for 5 min. The products of the digestion are separated by electrophoresis through a 1.5-mm 12%-polyacrylamide-SDS gel. After electrophoresis,

the gels are fixed in 10% acetic acid/40% methanol for 30 min, treated in EN 3 HANCE for an additional 30 min, and dried under vacuum for 2 h at 80°C. Autoradiography is then performed to visualize the radiolabeled digestion products.

EVALUATION

For evaluation of the protease digestion assay, the partially protease-resistant conformation product of PPAR γ is visualized by autoradiography on SDS-PAGE after incubation with the thiazolidinedione and increasing concentrations of trypsin.

K.6.4.4.4

Living Cell Luciferase Assay

The luciferase assays were used by Seimandi and coworkers (2005) to investigate the differential responses of PPAR α , PPAR δ and PPAR γ reporter cell lines to selective PPAR synthetic ligands. For this, cells are seeded at a density of 40,000 cells per well in 96-well white opaque tissue culture plates. Tested compounds are added 8 h later, and cells are incubated for 16 h. Experiments are performed in quadruplicate. At the end of incubation, culture medium is replaced by medium containing 3×10^{-4} M luciferin. Luciferase activity is measured for 2 s in intact living cells using a MicroBeta Wallac luminometer (Perkin-Elmer).

K.6.4.4.5

Lysed Cell Luciferase Assay

PROCEDURE

Cells are seeded at a density of 200,000 cells per well in 24-well test plates. Test compounds/drug candidates are added 8 h later, and cells are then incubated for 16 h. Cell extract preparation is done essentially as recommended by Promega. Compound-containing medium is removed, and cells are washed with PBS. Cells are lysed in 25 mM Tris/phosphate (pH 7.8), 2 mM EDTA, 1% Triton X-100, 2 mM DTT, 10% glycerol. Protein concentration is estimated with the Bradford assay kit and BSA as standard.

Cell protein extracts (100 μl) are loaded onto 96-well white opaque tissue plates, and luciferase activity is measured after injection of luciferase detection buffer (20 mM Tricine, pH 7.8, 1.1 mM (MgCO_3) 4.0 mM $\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.7 mM MgSO_4 , 0.1 mM EDTA, 33 mM DDT) and luciferase assay substrate (0.5 mM luciferin, 0.5 mM ATP, 0.3 mM coenzyme A)

using a luminometer (e. g. Berthold Technologies). Luminescence is measured for 2 s.

EVALUATION

In the transactivation assay, each compound is tested at various concentrations in at least two independent experiments. For each experiment, tests are performed in duplicate or triplicate for each concentration, and data are analysed as means and standard deviations. Individual agonist concentration-response curves, in the absence and presence of antagonist, are fitted using the sigmoidal dose-response function of a graphics and statistics software program (e. g. GraphPad Prism, version 4.0). EC₅₀ (effective concentration for half-maximal luciferase activity) and IC₅₀ (half-maximal inhibitory concentration) values are calculated from equations used to fit the data in this graphic software. Transactivation data are presented as EC₅₀ and IC₅₀ values for each compound tested. To analyze significance of PPAR isotype activation, PPAR agonist treatment is compared with controls using one-way analysis of variance (ANOVA) with the help of GraphPad Prism software.

In this assay system, the expression of PPAR isotypes differentially modulates the reporter gene basal activity and thus provides interesting information on the recruitment of cell type-specific coregulators by PPARs. Using specific PPAR agonists and antagonists, these cell lines permit an easy, rapid, and specific identification of subtype-selective PPAR ligands in a standardized microtiter plate assay. Furthermore, since HG5LN cells are putative host cells for other GAL4 nuclear receptor chimeras, RAR, thyroid hormone receptors, and steroid receptors, the assay system may be helpful to characterize the nuclear receptor selectivity of their nuclear receptor ligands which could also play important roles in the pathogenesis and therapy of metabolic diseases.

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K.6.4.5

Effect on Proliferation

PURPOSE AND RATIONALE

Both, insulin and IGF-1 stimulate growth and proliferation of cells which express the corresponding receptors at adequate number. However, they differ considerably in their relative potency with IGF-1 exerting at least 100-fold higher/lower mitogenic/metabolic activities compared to those of insulin. The *in vivo* metabolic action of IGF-1 can be compared with insulin in adult rats using the following methods (DeMeyts 1994, Ulrich and Schlessinger 1990, Schlessinger and Ulrich 1992, Simpson et al. 1998), (i) dose-dependence and time-dependence of blood sugar decrease after intravenous injection, (ii) the antilipolytic effect (decrease of non-esterified fatty acids) after i.v. injection, (iii) stimulation of glucose disposal dur-

ing euglycemic clamping after intravenous infusion. Furthermore, all other assays described for insulin can be applied. Gazzano-Santoro and coworkers (1998) described a cell-based potency assay for IGF-1.

PROCEDURE

Cell Culture

Cells from the human cell line HU-3, established from the bone marrow of a patient with acute megakaryoblastic leukemia, are adapted to grow in the presence of human thrombopoietin. Removal of thrombopoietin results in decreased proliferation and rapid loss of viability. Cells are cultured in RPMI-1640 medium with 2% heat-inactivated human serum, 2 mM glutamine, 10 mM HEPES (pH 7.2) and 5 ng/ml thrombopoietin in culture flasks. They are grown in suspension at 37°C in a humidified 5% CO₂ incubator and are routinely subcultured every two or three days when densities reach 0.8–1.5 × 10⁶ cells/ml.

Alternatively, the proliferative potency of insulin analogs can be determined by measuring the proliferation of human mammary epithelial cells (HMEC, Clonetics USA) in culture. HMEC obtained at passage 7 and expanded and frozen at passage 8 are used at passage 10 for all experiments. Cells are cultured in growth medium (fully supplemented MEGM, Bio Whittaker, 10 ng/ml hEGF, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 50 µg/ml gentamycin, 50 ng/ml amphotericin-B, 13 mg/ml bovine pituitary extract) which is changed every day. For a growth experiment, the medium is changed to assay medium lacking insulin but containing 0.1% BSA.

Assay

The HU-3 cell growth assay is performed under serum-free conditions in assay medium consisting of RPMI-1640 supplemented with 0.1% BSA, 10 mM HEPES (pH 7.2) and 50 µg/ml gentamycin. HU-3 cells are washed twice in the assay medium and resuspended at a density of 0.25 × 10⁶ cells/ml. In a typical assay, 100 µl of a cell suspension (25,000 cells/well) and 100 µl of IGF-1 at varying concentrations are added to flat bottomed 96-well tissue culture plates at 37°C and 5% CO₂ and cultured for 2 d. 40 µl of Alamar Blue™ (undiluted) are then added and the incubation continued for 7–24 h. The plates are allowed to cool to room temperature for 10 min on a shaker and the fluorescence is read using a 96-well plate fluorometer with excitation at 530 nm and emission at 590 nm.

The HMEC cell growth assay is performed in 96-well Cytostart scintillation microplates (Amerham Pharmacia). One day one, HMEC cells are seeded

at a density of 4,000 cells/well in 100 µl of assay medium. Insulin in the growth medium is replaced with graded doses of recombinant human insulin or insulin analog/compounds from 0–1 µM final concentration. After 4-h incubation, 0.1 µCi of ¹⁴C-thymidine in 10 µl of assay medium is added to each well and plates are read at 48 h and/or 72 h in a microtiter plate scintillation counter.

EVALUATION

Recombinant human insulin and IGF-1 are controls used in each assay run, and recombinant human insulin is on each assay plate. Results, expressed as relative fluorescence units, are plotted against IGF-1/compound concentrations using a 4-parameter curve-fitting program. Test compounds are compared with the standard. Typically, the maximal growth response is between 3- to 4-fold stimulation over basal. Response data are normalized to between 0 and 100% response equal to 100 X (response at concentration X – response at concentration zero) divided by (response at maximal concentration – response at zero concentration). Concentration-response data are fit by non-linear regression employing appropriate software. Analysis of the proliferative capacity of novel recombinant insulin analogs with improved kinetic profile in comparison to IGF-1 and insulin is of tremendous importance.

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K.6.5

Assays for Insulin and Insulin-Like Regulation of Energy Metabolism

K.6.5.1

Determination of Oxygen Consumption and Extracellular Acidification Rates

PURPOSE AND RATIONALE

Accumulating experimental evidence indicates that dysregulation of energy metabolism is a fundamen-

tal process that is associated with the phenotype of metabolic disorders, in particular type II diabetes and obesity. Many current anti-diabetic drugs (metformin, glitazones) target the pathways that control glucose metabolism. Very recently, a technologies (Bionas Inc. Germany, Seahorse Bioscience Inc. USA) have been developed that manages to rapidly profile the bioenergetic pathways in a variety of cell types. These include alterations caused by drugs that target the specific metabolic pathways the cell uses to ensure its energy demands, normal metabolic functions and survival. These technologies allow a more detailed analysis of the link between glucose/lipid metabolism and energy metabolism, including interference of one or the other by compounds/drug candidates, as well as the screening for or characterization of drug candidates affecting or leaving unaffected those pathways. For this, both the oxygen consumption rate (OCR), which primarily reflects mitochondrial respiration, and the extracellular acidification rate (ECAR), which primarily reflects lactic acid production (glycolysis), are measured using the Bionas or Seahorse technology. Furthermore, the OCR and ECAR readings allow to profile the metabolic sensitivities and degree of inhibition/stimulation of a number of cell lines relevant for the study of glucose and lipid metabolism and its regulation toward modulators of anaerobic and aerobic energy metabolism (e.g. phloretin, 2-deoxyglucose, dinitrophenol). Analysis of the sensitivities of the cell lines to these modulators provides insights into their bioenergetic preferences/dependencies and their global physiological responses to the modulation. This characterization may be useful for the selection of cell lines appropriate for use in screening for compounds with anti-proliferative activity (ECAR for anaerobic energy metabolism; Boros et al. 2002) as well as insulin-like metabolic activity (OCR for aerobic energy metabolism; Wolf et al. 1997), with regard to sensitivity and responsiveness of their energy metabolism and the relevant bioenergetic pathways (Ehret et al. 2001). During the last decade multiparametric cellular microelectronic interdigitated biosensor chips for microphysiological and screening applications with living cells have been developed (Ehret et al. 2001, Lehmann et al. 2000).

PROCEDURE

Bionas 2500 Analyzing System

The Bionas 2500 analyzing system is an automated system suitable for online cell-based compound/drug candidate identification and profiling. The innovative aspect of this bioanalytical device is the adaptation of

electronic chip design for a completely non-invasive and multiparametric sampling of metabolically relevant readouts in long-term tissue and cell culture. The system uses a silicon-based technology to continuously monitor minute changes in (i) solution pH resulting from the output of acid metabolites excreted by living cells into their immediate microenvironment (Lehmann et al. 2001), (ii) oxygen content resulting from oxygen consumed by living cells from their immediate microenvironment and (iii) electrical impedance resulting from the electrical isolation of intact cell membranes close to the sensor surface (adhesion/confluence) (Ehret et al. 1998). The Bionas 2500 analyzing system works in real-time, allowing the rapid and continuous monitoring of cellular activity and the measurement of recovery from a particular challenge and the process of desensitization.

Cells are seeded onto Bionas metabolic chips the day prior to the experiment and left to attach to the sensor surface overnight. The sensor chips are then transferred into the Bionas 2500 analyzing system. The system has the capacity to measure the acidification, respiration and adhesion/confluence rate from six separate sensor chips. Modified, low-buffered medium is pumped across the cells at a rate of 56 $\mu\text{l}/\text{min}$ during which time the pH and the oxygen content of the microenvironment surrounding the sensor is maintained constant. To measure the rates for acidification and respiration from cells the fluid flow is periodically halted, allowed a build-up of acid metabolites and a depletion of oxygen in the chamber and, therefore, an alteration in the fluid pH and oxygen content. Flow is resumed and the acid and oxygen-depleted fluid flushed out of the chamber. This flow-on, flow-off cycle is repeated throughout the experiment. To measure the adhesion/confluence, no fluid perfusion is necessary. After collecting baseline acidification, respiration and adhesion rates, the compound/drug candidate is introduced and the effect on acidification, respiration and adhesion rates are monitored.

Seahorse XF Instrument

Adherent 3T3-L1 adipocytes, L6-myoblasts/myotubes or HepG2 cells are seeded in 24-well Seahorse cell culture microplates at various cell densities per chamber per well. Approximately 45 min prior to the assay, the culture medium is exchanged with a low-buffered RPMI assay medium to ensure accurate ECAR readings. For detection of acute drug responses, OCR and ECAR are measured for 5 min in each well to establish a baseline. Compound/drug solution is then added and followed by measurement of OCR and ECAR.

For time resolved measurements, test measurements are made at a series of time points. Non-adherent cells (primary hepatocytes and adipocytes) are seeded onto Cell-Tak-coated Seahorse cell culture microplates either before or after the compound/drug treatment. Cell-Tak treatment of plates is performed according to manufacturers instruction. Cells remain attached to the surface of the well after the measurement as determined by light microscopy. Cells are lifted from the culture surface by simple pipetting without trypsin treatment.

A unique feature of the Seahorse instrument is its ability to make accurate and repeatable measurements in as little as 5 minutes. This is accomplished by isolating an extremely small volume (less than 10 μ l) of medium above the cell monolayer. Cellular metabolism causes rapid, easily measurable changes to the "microenvironment" in this small volume. Typically, a measurement cycle is performed for 2–10 min. During the time, analyte levels are measured every 8 sec until oxygen concentration drops approximately 10% and medium pH declines approximately 0.1 unit. The measurement is performed using fluorescent biosensors embedded in a sterile disposable cartridge that is placed above a 24-well tissue culture microtiterplate.

EVALUATION

Baseline metabolic rates are typically measured twice and are reported in nmol/min for OCR and mpH/min for ECAR. Compound/drug is then added to the medium and mixed for 5 min, and then the post-treatment OCR and ECAR measurements are made and repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR changes. Because these measurements are non-destructive, cells can be profiled over a period of minutes, hours or days.

K.6.5.2

Metabolomics

GENERAL CONSIDERATIONS

The flow of information from DNA to cellular building blocks is the central dogma of biology. DNA is transcribed into RNA, then translated to proteins, which then make small molecules. While there may be over 25,000 genes, 100,000 to 200,000 transcripts and up to 1,000,000 proteins, it is estimated that there may be as few as 2,500 small molecule species in the human metabolome. Metabolomics is the study of the metabolome, i.e. of the repertoire of these non-proteinaceous, endogenously-synthesized small molecules present in an organism. Representa-

tive small molecules include well-known compounds such as glucose, cholesterol, ATP and soluble signaling molecules, such as cAMP, AMP, diacylglycerol. These molecules are the ultimate product of cellular metabolism. The metabolome refers to the catalogue of those roughly 2,400 known endogenous metabolites in a specific organism. Most important, the normal physiological and disease states are ultimately manifest at the level of biochemistry and more precisely, at the level of the metabolome. It is more clear-cut and more quantitative because it is known where metabolites fit in biochemical pathways and the metabolites' relative concentrations are measurable. This makes a clear difference to many of the other "omics" sciences. It seems very likely that intelligent application of this methodology in appropriate animal disease models will considerably facilitate the identification and characterization of compounds/drugs for most indications and for metabolic diseases, in particular, with regard to potency, safety, accuracy, predictability and the time required. The putative "systems" contribution of metabolomics to pharmaceutical drug discovery and development has been reviewed by Harrigan (2006).

PURPOSE AND RATIONALE

Metabolomics technology determines the small molecule repertoire of a cell or a biological sample typically using a proprietary approach of simultaneous, multidimensional molecular analysis, followed by sophisticated data analysis and visualization. Biological samples are extracted using special protocols and the resulting extracts are separated and analyzed using a variety of detection techniques. Chromatographic outputs are analyzed using multiple mass spectrometers, which accurately identify the mass for a particular molecular species. Electrochemical detectors monitor additional broad classes of compounds. By choosing appropriate types of separation and detection technologies and mass spectrometers, the cellular metabolites are analyzed according to their various properties. Modified mass spectrometers produce data streams that are automatically processed and stored in proprietary information management systems. The data are quality-checked, reduced, refined and abstracted using custom algorithms and software. Data visualization is then used to compare experimental data to a large data base of control information. Hundreds of compounds of known identity are measured in a sample and contrasted to controls. Unique unknowns are brought to light for structure elucidation.

PROCEDURE

Metabolomics research is characterized, at least in part, by two different (but not necessarily mutually exclusive) conceptual approaches broadly defined as “targeted” and “non-targeted” (Harrigan and Goodacre 2003, Goodacre et al. 2003, Vaidyanathan et al. 2005). Non-targeted approaches provide a hypothesis-free global overview of high abundance metabolites most affected by experimental perturbation or disease. Targeted approaches which highlight identified and pre-selected metabolic pathways may prove more relevant in evaluating the impact of a compound/drug candidate on metabolic regulation. Technology platforms for targeted approaches are based on discrete optimized analytical strategies for different classes of metabolites or pathways. This approach represents both an accommodation with the wide differences in physicochemical structure, stability and differential abundance of metabolome components. It clearly facilitates greater evaluation of low-abundance, biologically important metabolites, such as eicosanoids, other signalling lipids and hormones. A key challenge for vendors providing instrumentation to support metabolomics is in establishing an optimal balance between the accuracy and range of metabolite measurements. Targeted metabolomics approaches additionally allow adoption of flux-based methodology where specifically designed tracer-labeled substrates can be incorporated into test biological systems and their distribution and metabolic fate recorded. Metabolic flux analyses provide an operational “moving picture” rather than a compositional “snapshot” of a biological system. At present, this approach is somewhat underrepresented in metabolomics but it may be pointed out that the data acquisition technologies used here are essentially that used in compositional studies. The data acquisition technologies utilised in metabolomics, primarily nuclear magnetic resonance spectroscopy and mass spectrometry have been extensively reviewed (Weljie et al. 2006).

Applications in Early Drug Discovery

There is considerable emphasis on metabolomic applications in later stages of drug discovery, including clinical trials. However, strategies to reduce attrition may be most cost-effectively implemented at the earliest stages of drug discovery, which encompasses disciplines such as target validation and high-throughput screening. In current drug discovery projects substrate/product ratios are used as mechanistic markers of enzyme function in increasing number. It is critical that a drug candidate be shown to act on its target

throughout all stages of the value chain and that modulation of that target does indeed impact disease progression.

Applications in the Analysis of Animal Models

The development of drugs for the regulation of metabolic diseases, such as cardiovascular diseases, obesity and type II diabetes, clearly requires considerable efficacy and toxicological testing in animal models. Efficacy assessments also require that regulation of metabolism in animal models is reasonably well understood. A metabolomics study utilising an analytical platform developed by Lipidomics Technologies Inc. that allows quantitative measurement of more than 500 lipid metabolites in blood and tissue samples has recently been employed to probe metabolic regulation in the low density lipoprotein receptor null (LDLRKO) mouse, a model for atherosclerotic progression (Krul et al. 2006).

Correlation of Metabolome, Proteome and Transcriptome Data Sets

An increasing area of interest is correlation of metabolomic data sets with proteomic and transcriptomic data sets (Davidov et al. 2004). While the goal of such studies is presumably to provide a deeper understanding of biological systems it may be worth pointing out that if the metabolome were truly closer to phenotype, then the use of complementary data from genomics, transcriptomics and proteomics could be perceived as merely ancillary or even potentially confounding. In an integrated transcriptomic and NMR-based metabolomic study of fatty liver in the rat, an inverse correlation between stearyl CoA desaturase mRNA levels and levels of unsaturated fatty acids described as “surprising” by the authors was revealed (Griffin et al. 2004). This observation is somewhat less surprising when placed in the context of a metabolomic demonstration that gene expression patterns in the adipose tissue of obese mice resulted in assessments of rates of fatty acid synthesis and lipogenesis that are inconsistent with direct measurements of the metabolic fluxes (Hellerstein 2003).

Future Metabolomics Technologies

The development of a new methodology for determining the concentration of protein-unbound FFA in intracellular and extracellular milieu entitled “Fluorescent Probes for Hydrophobic Metabolites” represents a particularly intriguing programme. Although FFA levels are clearly critical in many diseases, monitoring of

unbound FFA profiles remains technically challenging and improved detection methodologies can only help facilitate drug candidate progression in a range of therapeutic areas. The use of fluorescently labeled metabolite probes is also a theme in a proposal "Glycolipid Metabolism in Single Cells" to dissect glycolipid metabolism in single neurons. This proposal will focus on the development of different fluorescently labeled substrates within two different glycolipid metabolite pathways and offers particular promise in pharmacological evaluations of neuroactive agents. Another cellular neuro-metabolomic proposal "Technologies for Cellular Neurometabolomics" addresses the use of a suite of technology developments including unique sampling protocols, microfluidically-based sample conditioning unit with integrated electrophoretic separations, followed by native fluorescence and mass spectrometric detection and capture of appropriate metabolites into nl-volume capillaries for nl-volume NMR spectroscopic characterization.

EVALUATION

Companies (e. g. Metabolon Inc.) have developed proprietary software to link the resulting profile directly to metabolic pathway maps. This linkage greatly facilitates the identification of particular pathways that are affected in the biological sample relative to controls. In this way, the genes, RNAs, and proteins involved in a disease can be quickly and efficiently elucidated. By analyzing a sample using multiple mass spectrometry-based technologies, integrating the data, and analyzing through proprietary software and algorithms, the characterization of compounds/drugs in animal disease models, in general, for metabolic diseases such as type II diabetes, in particular, can be achieved much faster and more accurately than previously possible. Moreover, academic efforts have recently been initiated supporting well-conceived and technically feasible innovations that have the potential to drive metabolomics as a biologically informed science and provide metabolomic data that can be integrated into early drug candidate evaluation and also guide subsequent biomarker-enabled *in vivo* studies.

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K.6.6

Assays for the Expression and Release of Insulin and Glucose-Regulating Peptide Hormones from Pancreatic β -Cells

K.6.6.1

Insulin Release from the Isolated Perfused Rat Pancreas

PURPOSE AND RATIONALE

The *in vitro* perfusion of the isolated rat pancreas as described by Anderson and Long (1947), Ross (1972), Grodsky and coworkers (1983, 1984) and Muñoz and

coworkers (1995) offers the advantage to study the influence of carbohydrates, hormones and drugs such as sulfonyleureas not only on insulin but also on glucagon and somatostatin secretion without interference of secondary effects resulting from changes in hepatic, pituitary or adrenal functions.

PROCEDURE

The insulin concentration of the perfusate of the rat pancreas prepared as described (see K.5.4.1) is determined with the RIAGnost kit using rat insulin as standard in every second sample. The determination is done immediately after the end of an experiment.

K.6.6.2

Insulin Release from the Isolated Perfused Rat Pancreatic Islets

PURPOSE AND RATIONALE

An assay with isolated pancreatic islets to study the dynamic response and transitions between various metabolic states has been recommended by Idahl (1972). A further description and discussion of the method were given by Malaisse-Lagae and Malaisse (1984).

PROCEDURE

The insulin concentration of the perfusate of the rat islets prepared as described (K.5.4.2) is determined with the RIAGnost kit using rat insulin as standard in every second sample. The determination is done immediately after the end of an experiment.

EVALUATION

The raw data are expressed in μU insulin per islet/min. Mean and standard error of the mean of each time interval are calculated for graphical representation. The values under exposure to drug are compared with the values under perfusion with glucose only, and with the effect of elevated glucose.

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K.6.6.3

Insulin Release from Cultured β -Cells

PURPOSE AND RATIONALE

Insulin released into the supernatant of monolayers of cultured β -cells grown in 96-well culture dishes as described above is determined radioimmunochemically (see above) using a guinea pig anti-rat insulin antibody, ^{125}I -labeled human insulin as tracer and rat insulin as standard. Free and bound radioactivity is separated using an anti-IgG (goat anti-guinea pig) antibody. Typically, the sensitivity of this assay is 17 pM, and the coefficient of variation is less than 3% at both low and high levels.

PROCEDURE**Insulin Secretion from MIN6 Cells**

Cultured MIN6 cells (1×10^5) are replated and cultured (see K.5.4.4) for 24 h before being used in secretion assays. Insulin secretion is measured during 1 h static incubations in KRBH (118.5 mM NaCl, 2.54 mM CaCl_2 , 1.19 mM KH_2PO_4 , 4.74 mM KCl, 25 mM NaHCO_3 , 1.19 mM MgSO_4 , 10 mM HEPES, pH 7.4) containing 0–20 mM glucose (basal or glucose-induced secretion) and/or compounds/drugs. Samples of the supernatant are assayed for insulin. To determine total insulin content, insulin is extracted using 95/5 ethanol/acetic acid. Insulin is measured using a mouse insulin ELISA kit.

Insulin Secretion from RINm5F Cells

One day prior to the experiment, approximately 1×10^6 RINm5F cells, grown as described above, are seeded into 24-well test plates. At the time of the experiment, the culture medium is aspirated, and the cells are washed with 1 ml of a modified KRBH containing 10 mM HEPES/KOH (pH 7.4), 5 mM NaHCO_3 , 0.5% BSA and 0–20 mM glucose. After pre-incubation for 30 min at 37°C with the above buffer, compounds/drug candidates dissolved in DMSO at 10 mM are added (final concentration range from 10 nM to 100 μM) and incubated for 30 min at 37°C. The incubation is stopped by aspiration of the buffer which is stored at -80°C until measurement of insulin by ELISA or RIA. After the aspiration, the cells are washed with PBS and dissolved in 0.5 ml NaOH (0.1 M). After overnight incubation, the resulting solution is collected for assay of cellular protein content.

Insulin Secretion from INS1 Cells

INS1 cells (5×10^4 per well in 12-well plates) are treated with RPMI 1640 medium containing 3–20 mM glucose and 0.5 mM FFA or a mixture of oleic and palmitic acids in equal ratio, with a total concentration of 0.5 mM. Control wells contained 0.5% fatty acid-free BSA in medium. After 24 h, the cells are washed in PBS and pre-incubated for 2 h in KRBH containing 0–20 mM glucose (119 mM NaCl, 4.75 mM KCl, 5 mM NaHCO_3 , 1.2 mM MgSO_4 , 1.18 mM KH_2PO_4 , 20 mM HEPES, pH 7.4, 2.54 mM CaCl_2). Glucose responsiveness is evaluated by an initial 30-min incubation in KRH containing 3 mM glucose followed by incubation for 30 min in KRH containing 20 mM glucose. Samples are collected for insulin measurement using RIA or ELISA with CV 4.5% and minimal detection 0.15 $\mu\text{g/l}$.

Insulin Secretion from Attached INS-1E Cells

The secretory responses to glucose and other secretagogues are tested in INS-1E cells between passages 54–95 according to the procedure of Merklen and coworkers (2004). Before the experiments, cells are maintained for 2 h in glucose-free culture medium. The cells are then washed twice and preincubated for 30 min at 37°C in glucose-free KRBH (135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO_3 , 0.5 mM NaH_2PO_4 , 0.5 mM MgCl_2 , 1.5 mM CaCl_2 , and 10 mM HEPES, pH 7.4) BSA (0.1%) is added as an insulin carrier. Next, cells are washed once with glucose-free KRBH and then incubated for 30 min in KRBH and compounds/drug candidates. Incubation is stopped by putting the plates on ice. The supernatants are collected for insulin secretion, and cellular insulin contents are determined from acid-ethanol extracts. Insulin secretion is measured by RIA or ELISA using rat insulin as standard (see K.4.1).

Perfusion of INS-1E Cell Clusters

Spheroid clusters composed of INS-1E cells are preincubated for 2 h in glucose-free culture medium. After low speed centrifugation (500 rpm), cell clusters are resuspended in glucose-free KRBH and counted, and about 500 spheroids are distributed per chamber in a 250- μl volume thermostated at 37°C. The flow rate is set at 0.5 ml/min, and fractions are collected every minute after a 20-min washing period at basal 2.5 mM glucose. At the end of the perfusion, cell clusters are collected, and insulin contents are determined from acid-ethanol extracts.

Total Insulin Content

Cells from 4 wells of 24-well culture dishes are scraped into ice-cold PBS containing 5.5 mM glucose and 0.1% fatty acid-free BSA and immediately frozen in liquid N_2 . Frozen cells are sonicated in acidic ethanol (0.2 M HCl in 87.5% ethanol). The samples are then centrifuged. The supernatant is used for measurement of total insulin content. Insulin in the perfusion fractions and cell extracts is determined by RIA or ELISA (see K.4.1).

K.6.6.4**Lipolysis in β -Cells****PURPOSE AND RATIONALE**

Besides the *de novo* synthesis of LC-CoA starting with the ACC reaction and malonyl-CoA formation, leading to inhibition of the translocation of LC-CoA into mitochondria, lipid-derived signaling molecules involved

in insulin secretion may also originate from the breakdown of TAG stores in β -cells. This may occur through activation of HSL, the enzyme that exerts a major control in TAG hydrolysis in adipose tissue (for a review see Yeaman 2004).

It was first demonstrated in 1999 that HSL is expressed and active in the β -cells of the pancreatic islet (Mulder et al. 1999). Two isoforms are expressed in the β -cell, the 84-kDa protein seen in adipocytes and other tissues and an 89-kDa protein (Mulder et al. 1999) encoded by exons 1–9 *plus* exon A, which is spliced to exon 1, introducing an additional 43 amino acids into the polypeptide (Lindvall et al. 2004).

The role of HSL in the β -cell has yet to be fully established. Two studies have independently looked at insulin secretion in HSL-knockout mice (Roduit et al. 2001, Fex et al. 2004). Both demonstrate features of insulin resistance in peripheral tissues, but differ in the effect of the knockout on insulin secretion from the pancreas. In one study, the glucose-responsiveness of insulin secretion was impaired (Roduit et al. 2001), both *in vivo* and in pancreatic islets isolated from the mice. The islets from the HSL knockout mice were totally unresponsive to glucose, but showed a normal response to depolarizing concentrations of KCl. This work is consistent with a key role for HSL in coupling of glucose metabolism to increased insulin secretion, perhaps by generating a lipid messenger such as DAG or fatty acyl-CoA from intracellular stores of TAG (Mulder et al. 1999, Roduit et al. 2001, Yaney et al. 2000). Also consistent with this role is the increased expression of HSL in response to high concentrations of glucose, with the elevated glucose ensuring an appropriate supply of the lipid mediators (Sorhede-Winzell 2001). The second study in HSL null mice demonstrated that insulin secretion was essentially unimpaired, with an increase in islet mass being found, presumably as a result of the insulin resistance in peripheral tissues (Fex et al. 2004). Possible reasons for the apparent discrepancies between the two studies may rely on the genetic background of the mice.

However, recent studies on transgenic mice that overexpress HSL specifically in the β -cell have provided evidence for a role for HSL in mediating the lipotoxicity associated with type II diabetes. These mice showed impaired glucose tolerance and had defective GSIS when fed a high-fat diet, accompanied by lower levels of β -cell TAG than found in control mice fed the same diet (Sorhede-Winzell 2003a). One proposed mechanism for this is that HSL provides endogenous ligands for lipid-activated transcription fac-

tors, including the PPAR-activated receptors. In this regard, it would appear that, in control animals, the accumulation of intracellular TAG is actually a protective mechanism (Listenberger et al. 2003), with the lipotoxicity being manifested when the storage capacity is exceeded or when the lipid stores are mobilized by HSL. Consistent with this is the observation that long-term feeding with a high-fat diet leads to down-regulation of pancreatic HSL levels by as much as 75%, presumably as a protective mechanism against the generation of these lipid metabolites (Sorhede-Winzell 2003b).

PROCEDURE

Lipolysis in cultured β -cells is determined as the release of glycerol into the incubation medium. Since FFA and glycerol are the end-products of lipolysis and glycerol is not metabolized by the β -cell due to low expression of glycerol kinase, glycerol release actually reflects the degree of lipolysis in cultured β -cells. Cells grown in 24-well culture dishes are incubated in 500 μ l of RPMI 1640 medium containing 10% (by vol.) heat-inactivated fetal calf serum, 50 IU/ml penicillin, 0.25 μ g/ml amphotericin B and 50 μ g/ml streptomycin in the presence of increasing concentrations of glucose (2.8–16.7 mM) at 37°C for up to 4 h in an atmosphere of humidified air/CO₂ (19/1). After certain periods of time, 20- μ l portions are transferred to a 96-well culture plate and incubated with glycerol kinase (50 U/ml) and luciferin/luciferase reagent. After addition of 10 μ l of 1 mM ATP, the luminescence is measured in a luminometer after 4, 8 and 12 min. The glycerol concentration is calculated from the standard curve.

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K.6.6.5

Measurement of Ca^{2+} Levels

Imaging

For imaging of cultured β -cells according to the protocol of Toye and coworkers (2005) with the modifications adapted from Freeman and coworkers (2006), MIN6 cells are cultured on 35-mm Fluorodishes (World Precision Instruments) and incubated with DMEM *plus* 3 μM Fura-2-AM (Molecular Probes) for 40 min at 37°C. They are imaged at room temperature (20–24°C) using an IonOptix fluorescence system with 340 nm and 380 nm dual excitation. The 510 nm emission ratio is collected at 1 Hz. Background subtraction is performed by measuring fluorescence from a cell-free region in the field of view. Cells are perfused continuously with extracellular solution containing 137 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl_2 , 1.2 mM MgCl_2 , 10 mM HEPES/NaOH (pH 7.4) *plus* glucose or compounds/drug candidates.

Fluorometry

Calcium levels are monitored in cells transduced the day before the experiment with the calcium-sensitive photoprotein aequorin. Measurements of mitochondrial and cytosolic calcium are performed using the corresponding adenovirus constructs, AdCA-mtAeq and AdCA-cyAeq, respectively. The 2-h preincubation period also serves to load cells with the aequorin prosthetic group, i.e. 5 μM coelenterazine [2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*b*]pyrazin-3-(7H)-one; Calbiochem].

Calibration is calculated based on the total counts obtained at the end of the trace following permeabilization of the cells with 50 μM digitonin and 10 mM CaCl_2 exposure.

K.6.6.6

Measurement of $^{86}\text{Rb}^+$ Efflux

PURPOSE AND RATIONALE

The binding of a sulfonylurea to its receptor SUR1, a subunit of the ATP-sensitive K^+ channel of β -cells, leads to blockade of the channel activity and concomitant decrease in the K^+ efflux (Boyd III et al. 1991). The resulting depolarization of the β -cell plasma membrane causes opening of the voltage-gated Ca^{2+} channel (Nelson et al. 1987, Niki et al. 1989, Rorsman and Trube 1986, Nichols et al. 1996). The rise in the intracellular $[\text{Ca}^{2+}]$ triggers the exocytosis of insulin. $^{86}\text{Rb}^+$ efflux can be used as marker for K^+ efflux and analyzed in response to treatment of cultured β -cells with compounds/drug candidates.

PROCEDURE

RINm5F cells are grown and plated at a density of 200,000 cells/well (24-well tissue culture plates). $^{86}\text{Rb}^+$ efflux experiments are performed at 37°C and overnight equilibration of cells in RPMI 1640 medium supplemented with 10% FCS, 0.1 $\mu\text{Ci/ml}$ $^{86}\text{RbCl}$ and 0.2 $\mu\text{Ci/ml}$ L- ^3H]leucine (internal marker of cell recovery). After removing the medium, cells are preincubated in a medium containing 120 mM NaCl, 1.8 mM CaCl_2 , 0.8 mM MgCl_2 , 10 mM KCl, 20 mM HEPES/NaOH (pH 7.5) supplemented with 0.1 μCi $^{86}\text{RbCl}$, 0.24 mg/ml oligomycin, 1 mM 2-deoxy-D-glucose and several concentrations of compounds/drug candidates as well as sulfonylureas (as positive controls). $^{86}\text{Rb}^+$ efflux studies are initiated by removing the pre-incubation medium and incubating the cells with 200 μl of the same medium/well without $^{86}\text{Rb}^+$, oligomycin, and 2-deoxy-D-glucose. Efflux is stopped by removing this medium and washing the cells three times with 1 ml of 0.1 M MgCl_2 at 37°C. Cells are extracted with 2 \times 1 ml of 0.1 N NaOH and counted. Total intracellular concentrations of ATP are measured after extracting the cells with 1% Triton X-100 by using the luciferase-luciferin technique.

EVALUATION

Inhibition is measured as percent of maximum $^{86}\text{Rb}^+$ efflux. Half maximum inhibition constants can be calculated.

MODIFICATIONS OF THE METHOD

Daniel and coworkers (1991) recommended a high through-put ^{86}Rb efflux assay in human medulloblastoma cells TE671 for screening of potassium channel modulators. Hu and coworkers (1995) cloned a voltage-regulated K channel from human hippocampus for transfection into CHO cells. The authors recommended this method as a high through-put assay to identify isotype-specific K-channel modulators.

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responses such as the release of hormones and neurotransmitters, or muscle contraction.

Studies on isolated cells and tissues, and more recently on genetically modified mice and patients with mutations in K_{ATP} channel genes, have demonstrated that K_{ATP} channels play a multitude of physiological roles (Seino and Miki 2004). They contribute to glucose homeostasis by regulating insulin secretion from pancreatic β -cells (Ashcroft and Rorsman 1989, Henquin 2000, Gribble and Reimann 2003, Rorsman et al. 1994, Koster et al. 2000, Seghers et al. 2000, Dunne et al. 2004), glucagon secretion from pancreatic α -cells (Gopel et al. 2000a), somatostatin secretion from D cells (Gopel et al. 2000b), and GLP-1 secretion from L cells (Gribble et al. 2003). In ventromedial hypothalamic neurons they mediate the counter-regulatory response to glucose (Miki et al. 2001), and in arcuate nucleus neurons they may be involved in appetite regulation. In these glucose-sensing cells, K_{ATP} channels respond to fluctuating changes in blood glucose concentration. In many other tissues, however, they are largely closed under resting conditions and open only in response to ischemia, hormones, or neurotransmitters. In cardiac muscle and central neurons the resulting reduction in electrical activity helps protect against cardiac stress and brain seizures (Zingman et al. 2002, Yamada et al. 2001). K_{ATP} channels are involved in ischemic preconditioning in heart (Gumina et al. 2003) and the regulation of vascular smooth muscle tone (opening of K_{ATP} channels leads to relaxation) (Daut et al. 1994, Chutkow et al. 2002). Given their critical role in regulating electrical excitability in many cells, it is perhaps not surprising that disruption of K_{ATP} channel function can lead to disease. To date, mutations in K_{ATP} channel genes have been shown to cause neonatal diabetes (Gloyn et al. 2004, Magge et al. 2004), hyperinsulinemia (Dunne et al. 2004, Glaser et al. 2000, Thomas and Lightner 1996, Huopio et al. 2000). The effect of compounds/drug candidates on the open/closed state of K_{ATP} channel and the electrical activity of β -cell plasma membranes can be assayed with the following assays, which may be helpful for the identification of insulin secretagogues and sensitizers for GSIS as potential drug in the anti-diabetic therapy.

K.6.6.7**Measurement of Cell Membrane Potential****PURPOSE AND RATIONALE**

ATP-sensitive potassium (K_{ATP}) channels couple cell metabolism to electrical activity of the plasma membrane by regulating membrane K^+ fluxes (for a review see Ashcroft 2005). A reduction in metabolism opens K_{ATP} channels, producing K^+ efflux, membrane hyperpolarization, and suppression of electrical activity. Conversely, increased metabolism closes K_{ATP} channels. The consequent membrane depolarization stimulates electrical activity and may thereby trigger cellular

PROCEDURE

Rat insulinoma cells RINm5F (Gögelein et al. 1998) or cultured pancreatic β -cells from NMRI mice (Rorsman and Trube 1985, Zünkler et al. 1985) or HIT T15 β -cells (Niki et al. 1989) are used. Whole cell patch-clamp experiments are performed according to Hamill and coworkers (1981), Rajan and coworkers

(1993), Lindau and Neher (1988) at room temperature with cells bathed in an external solution of 140 mM NaCl, 4 mM KCl, 10 mM HEPES (pH 7.4). The patch pipettes are mounted on a suction pipette holder. The outlet is connected to silicon rubber tubing through which the suction is applied. The pipette solution contains 135 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM HEPES (pH 7.5), 0.3 mM NaATP. Pipettes are prepared by pulling from borosilicate glass, coated with silicon rubber, and heat polished at the tip. Pipette resistances are ranging between 4 and 7 MΩ. A patch-clamp amplifier (e. g. EPC 7, List Electronic, Darmstadt, FRG) is used which allows capacitance and series resistance compensation. The series resistance after achieving the whole cell recording configuration is 5–20 MΩ and 50% series resistance compensation is used to keep the voltage error during current flow below 2 mV. The cell membrane potential is held at –70 to –80 mV and hyper- and depolarizing voltage pulses of 10 mV amplitude are applied alternatively every 2 s. Voltage-dependent currents, i. e., the Ca²⁺ inward current and the delayed K⁺ outward current are not activated by these low pulse amplitudes and, therefore, most of the current is flowing through ATP-dependent K⁺ channels. Inside-out patches are prepared according to Hamill and coworkers (1981) by disrupting the outer vesicle membrane.

EVALUATION

The current and voltage signals are stored on magnetic tape or filtered by a 4-pole Bessel filter and displayed on a digital oscilloscope. Outward currents flowing from the cell to the bath are indicated by upward deflections and inward currents by downward deflections. Half maximal effective concentrations are calculated as EC₅₀ values.

MODIFICATIONS OF THE METHOD

Henquin and Meissner (1984), Henquin and coworkers (1984, 1985), Meissner (1990) measured membrane potential of mouse pancreatic β-cells. Hu and coworkers (2000) compared the effects of netaglinide, a non-sulfonylurea hypoglycemic agent, with sulfonylureas on pancreatic β-cell K_{ATP} channel activity with the whole-cell configuration of the patch-clamp technique in primary cultures of rat pancreatic β-cells. Wang and Giebisch (1991) reported a dual modulation of the renal ATP-sensitive K⁺ channel by PKA and PKC in cells of the cortical collecting duct of rabbit kidneys using the cell-attached and inside-out modification. Shieh and coworkers (2000) characterized K_{ATP} channel opener-activated currents in

pig and guinea pig bladder smooth muscle cells using the whole-cell patch-clamp technique. Shindo and coworkers (2000) examined with the whole-cell configuration of the patch-clamp technique the effects of a vascular relaxing agent on the heterologously expressed pancreatic-type ATP-sensitive K⁺ channels SUR1/Ki6.2, SUR2A/Ki6.2, and SUR2B/Ki6.2 in human embryonic kidney 293T cells. Using whole-cell and single-channel patch-clamp recording, Gomora and Enyeart (1999) studied pharmacological properties of a cyclic AMP-sensitive potassium channel, I_{AC}, which is distinctive among K⁺ channels both in its activation by ATP and inhibition by cyclic AMP. Alternatively, Maechler and coworkers (2004) monitored the cell membrane potential using 100 nM of the fluorescent probe bis-oxonol [bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; Molecular Probes]. Filters used for excitation and emission had wavelength optima at 544 and 590 nm, respectively.

K.6.6.8

Measurement of Mitochondrial Membrane Potential

PROCEDURE

Mitochondrial membrane potential ($\Delta\psi_m$) is measured using the fluorescent probe, rhodamine 123. Cells cultured in 24-well plates are maintained for 2 h in 2.5 mM glucose medium at 37°C before loading with 10 μg/ml rhodamine 123 (Molecular Probes) for 20 min at 37°C in KRBH. The $\Delta\psi_m$ is monitored with excitation and emission filters set at 485 and 520 nm, respectively. Glucose (additions on top of the basal concentration of 2.5 mM) and then the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) is added to each well.

K.6.6.9

Measurement of cAMP Production

Approximately 1×10^6 cultured β-cells (grown as described above) in 0.45 ml buffer (113 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 10 mM HEPES, pH 7.4, 2.5 mM CaCl₂ and 1.2 mM MgSO₄) containing 1% human serum albumin are pre-incubated for 10 min at 37°C, and then incubated for 10 min after the addition of 2 μl of IBMX (50 mM) in order to prevent the breakdown of cAMP. The reaction is then started by the addition of 50 μl of a peptide solution dissolved in the above buffer (final concentration range from 10 pM to 1 μM). After incubation for 10 min at 37°C, the reaction is stopped by the addition of 200 μl of 12% TCA. The reaction mixture is sonicated for 30 s at

25 W (e. g., Heat system, Ultrasonics) and centrifuged ($11,500 \times g$, 2 min). HCl (25 μ l, 1 M) is added to 0.5 ml supernatant. TCA dissolved in the supernatant is removed by diethyl ether (3×1 ml) and the resulting supernatant is stored at -80°C until cAMP assays being performed by use of a RIA kit.

K.6.6.10

Measurement of Cytosolic ATP Levels

Cytosolic ATP levels are monitored in cells expressing the ATP-sensitive bioluminescent probe luciferase 1 d after transduction with the specific AdRIP-Luc viral construct according to Maechler and coworkers (1998) and Rubi and coworkers (2001). After the preincubation steps described above, the 24-well plates are transferred to the plate reader in the luminometer mode. The luciferase substrate, 100 μ M beetle luciferin (Promega), is added to the KRBH. After a 10-min period in basal 2.5 mM glucose, cells are stimulated with the indicated glucose concentrations, and 20 min later the mitochondrial poison NaN_3 (2 mM) is added (as a control).

K.6.6.11

Analysis of Lipotoxicity

GENERAL CONSIDERATIONS

There is increasing experimental evidence that sustained elevated plasma and intracellular lipid concentrations result in impaired insulin action and secretion *in vivo* and *in vitro*. This has implications for the causal relationship between the pathogenesis of obesity and type II diabetes (Carpentier et al. 2001, Dobbins et al. 2002). It is known that increased plasma FFA concentrations reduce insulin-mediated glucose uptake and metabolism in muscle tissue presumably *via* the glucose-fatty acid (Randle) cycle (Randle et al. 1963), but the exact mechanism of action of FFA on the β -cell to modulate glucose-stimulated insulin secretion (GSIS) (Zhou and Grill 1994, Roche et al. 1998, Unger and Orci 2002) remains to be elucidated. A complex interrelationship between the metabolism of glucose and lipids in β -cells has been described (Prentki and Corkey 1996), and this critically depends on the duration of exposure (acute or chronic) to the lipid metabolites (Zhou et al. 1994, Roche et al. 1998). In conclusion, it is now generally accepted that in rodent β -cells and islets lipids and/or lipid precursors/metabolites interfere with GSIS and β -cell functionality (Maedler et al. 2001, El Assaad et al. 2003, Cnop et al. 2001), but their nature (e. g. TAG, FFA, acyl-CoA, DAG) and molec-

ular mode of action still remains a matter of intense research and debate.

However, serious doubts have been raised against the hypothesis of FFA-induced impairment of GSIS and β -cell death as playing a major role in the pathophysiology of human type II diabetes. Although increased plasma FFA and lipid (as lipoprotein TAG) concentrations and alterations in their composition occur in obesity in humans (Wang et al. 2003), this condition is not invariably linked to the onset of type II diabetes. Fewer than 12% of obese subjects with elevated lipid profiles suffer from diabetes (Ford et al. 2002). Furthermore, the concept of glucolipotoxicity (Poitout and Robertson 2002) proposes that, in type II diabetes, the elevation of both glucose and FFA could contribute to the decrease in pancreatic islet function as a result of both β -cell apoptotic death and decreased insulin secretion.

PURPOSE AND RATIONALE

The following assay systems enable the determination of effects of compounds/drug candidates on the fatty acid-induced (and glucose-induced) modulation of GSIS, cell death and TAG concentration, composition and localisation in cultured β -cells. They may be helpful for the identification and characterization of compounds/drugs with the potential of abrogating the lipo(gluco)toxic effects of lipid (metabolites) in β -cells in response to certain (patho)physiological stimuli (e. g. stress, reactive oxygen species, see K.8.8).

PROCEDURE

Viability Assay

INS1 cells are plated into 8-well Permanox slides (Gibco) and treated for 24 h with 0.1, 0.25, 0.5 and 1 mM of FFA in 3 or 20 mM glucose. Adjustments to the stock solution are made to ensure a constant BSA concentration. Following incubation, cells are rinsed in PBS, incubated for a further 30 min in PBS containing 10 μ M calcein-AM (Sigma) at 37°C and fixed in 2.5% paraformaldehyde for 30 min. Slides are rinsed twice more in PBS and the slides mounted in Vectorshield containing propidium iodide (Vector Laboratories). Viability is quantified by counting the propidium-iodide-positive nuclei and calcein-AM-positive cells (average cell count = 60 cells per field) and determining the percentage of propidium-iodide-positive nuclei in at least three fields of view per condition.

TAG and Phospholipid Content and Composition

INS1 or COS7 cells are seeded into 6-well plates at 2×10^5 cells per well and treated for 24 h with 0.5 mM

specific fatty acids. Total lipids are extracted from cells in chloroform/methanol (2/1) and lipid fractions are separated using solid-phase extraction (Burdge et al. 2000). The TAG and phospholipid fractions are collected and fatty acid methyl esters prepared using methanolic sulphuric acid. The fatty acid methyl esters are separated by gas chromatography (e.g. Agilent 6890 GC equipped with a DB-Wax 30 m capillary column coated with a polyethylene glycol stationary phase, internal diameter 0.25 mm, film thickness 0.25 µm; Agilent Technologies). Fatty acids are separated according to fatty acyl carbon chain length and degree of saturation. Intact TAG analyses are performed by high-temperature gas chromatography (HTGC; e.g. Hewlett Packard 5890 series II GC with a DB1-HT 15 m fused silica capillary column, internal diameter 0.32 mm, film thickness 0.1 mm). Hydrogen is the carrier gas and a flame ionisation detector is employed to monitor column eluent. HTGC analyses separates the TAG according to their carbon number. The absolute concentrations of individual fatty acids and TAG are determined typically by reference to internal standards (heptadecanoic acid [17:0] for NEFA; tripentadecanoin [15:0] for TAG; phosphatidylcholine, diheptadecanoyl [17:0] for phospholipid).

Light Microscopy

INS1 and cells are incubated in 8-well Permanox or glass slides (Gibco) with compounds/drug candidates for 24 h in the absence or presence of 3 or 20 mM glucose and 1 mM FFA. Cells are washed in PBS and fixed in 2.5% PFA and then stained for lipids. A stock solution of 0.5% (saturated) oil red O in propan-2-ol is freshly diluted (three parts stock solution in two parts distilled water) and filtered immediately before use. Cells are stained for 30 min at 37°C, then briefly destained in 60% isopropanol and washed in water before mounting in PBS/glycerol. A freshly prepared 1% Nile blue-solution in distilled water is filtered and an aliquot is further diluted in water to 0.02%. Fixed cells are washed twice in PBS. Nile blue solution (1%) is added to the cells for 10 min at 65°C or 70°C, after which the cells are washed in water and destained in 1% acetic acid (65°C) for 30 s. Cells are restained for 15 min in 0.02% Nile blue at 65°C or 70°C before being washed in water and mounted in PBS/glycerol.

Electron Microscopy

INS1 cells for morphological analysis are incubated with compounds/drug candidates in 25-cm² flasks for 24 h as described above. Cells are removed and gen-

tly prepared as a pellet, fixed in 2.5% glutaraldehyde (minimum 1 h) at room temperature (or for a 10-min period at 70°C), post-fixed in 2% osmium, dehydrated and embedded in Spurr resin. Cells for immunoelectron microscopy are fixed in ice-cold 4% paraformaldehyde (10 min) followed by 8% paraformaldehyde at room temperature (50 min), washed in PBS, dehydrated in methanol and embedded in LR Gold resin. Ultrathin sections are cut onto nickel grids and contrast-enhanced with uranyl acetate and lead citrate. Sections are examined in an electron microscope with an accelerating voltage of 80 kV.

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K.6.6.12

Interaction with β -Cell Plasma Membranes and K_{ATP} Channels

It is now established that increasing the closed probability of the β -cell ATP-dependent potassium channel (K_{ATP}) is the major mechanism through which sulfonylureas as well as glucose (*via* transport, phosphorylation and metabolism for generation of ATP) stimulate insulin release from pancreatic β -cells (for reviews see Ashcroft and Ashcroft 1992, Ashcroft 2005, Bryan and Aguilar-Bryan 1997, Gribble and Reimann 2003, Henquin 2000, Inagaki et al. 1996). The resulting reduction in potassium ion efflux causes depolarization of the β -cell plasma membrane which in turn leads to opening of voltage-sensitive Ca²⁺ channels of the L-type. The increased influx of Ca²⁺ and thus the elevated cytosolic Ca²⁺ levels trigger the fusion of insulin-containing secretory granules with the plasma membrane, presumably mediated by Ca²⁺/calmodulin-dependent protein kinase and additional unknown mechanisms (Philipson 1995, Aguilar-Bryan and Bryan 1999). The β -cell K_{ATP} consists of the regulatory sulfonylurea receptor subunits (SUR), such as SUR1, and the physically associated pore-forming potassium ion inwardly rectifying subunit, KIR6.2, in a multimeric assembly with the SUR and KIR6.2 subunits in 1:1-stoichiometry and four identical SUR/KIR6.2 complexes per functional channel unit (size of the channel holocomplex [SUR/KIR6.2]₄ about 1000 kDa; Skeer et al. 1994, Clement et al. 1997a and b, Bryan and Aguilar-Bryan 1999, Shyng and Nichols 1997). SUR1, which is predominantly expressed in neuronal/pancreatic β -cells, is an ATP-binding cassette (ABC) protein or transport ATPase, which closely resembles members of the multidrug resistance associated protein family with 17 predicted transmembrane domains (TMD) and two nucleotide-binding folds (NBF), which bind specifically (Mg²⁺)/ATP/ADP (Bryan et al. 1995, Inagaki et al.

1995 and 1996, Ueda et al. 1999, Gribble et al. 1997). The KIR6.2 subunits have two TMDs which somehow contribute to the K⁺ conductivity and selectivity (Babenko et al. 1998). The glibenclamide-binding site of SUR1 is proposed to consist of a benzamido (meglitinide)-binding site on TMDs 1–5 and the sulfonylurea (tolbutamide)-binding site on TMDs 12–17 based on photolabeling with [¹²⁵I]iodoglibenclamide and chimeric receptors (Ashfield et al. 1999, Babenko et al. 1999, Chutkow et al. 1996, Isomoto et al. 1996, Tanabe et al. 1999, Aguilar-Bryan et al. 1995). Interaction with both the benzamido- and sulfonylurea-binding sites could account for the several thousand-fold increase in affinity of glibenclamide vs. tolbutamide. SUR2A/B are splice variants of a single SUR2 gene differing in 42 amino acids at the carboxy-terminus, exclusively, with SUR2A expressed mainly in cardiac/skeletal muscle cells (Chutkow et al. 1996, Inagaki et al. 1996) and SUR2B in vascular/non-vascular smooth muscle cells and brain (Isomoto et al. 1996, Aguilar-Bryan et al. 1998). K⁺-channel openers (KCOs) bind to SURs at the tolbutamide-binding site encompassing those (flanking) TMDs 12–17 which surround the central core region of the tolbutamide-binding site (Uhde et al. 1999). SURs define the sensitivity of the K_{ATP} channel holocomplex for its sensitivity toward both sulfonylureas and KCOs with SUR1 mediating high sensitivity for sulfonylureas/low sensitivity toward KCOs and *vice versa* SUR2A/2B mediating low sensitivity for sulfonylureas/high sensitivity for KCOs (for a review see Ashcroft 2005).

The following assays enable the investigation of the direct binding of compounds/drug candidates to the K_{ATP} channels and their SUR subunits, which may be helpful for the identification of insulin releasers acting like sulfonylureas or glinides, which have been used successfully in the anti-diabetic therapy since decades.

K.6.6.12.1

Isolation of Membranes

PROCEDURE

For receptor binding studies, membranes are isolated from adenoma tissue suspension, cerebral cortex of Wistar rats (Kaubisch et al. 1982, Geisen et al. 1985) or from RINm5F cells (Müller et al. 1994a).

Cerebral Cortex Homogenates

The homogenates are prepared from decapitated Wistar rats. The cortices are immersed in ice-cold buffer, pH 7.5, and are homogenized under cooling in an Ultraturax tissue homogenizer. The cell membranes are

centrifuged ($50,000 \times g$, 1 h, 4°C) resuspended in fresh phosphate buffer and recentrifuged at the same speed. The resulting pellet is suspended in phosphate buffer, the final dilution being 1:50, based on wet weight of the cerebral cortex. One ml aliquot of this cell suspension is used for the incubations.

β -Cell Adenoma Tissue

The tissue is dissected from rats of the strain NEDH, is homogenized in 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 0.5 mM EDTA, 100 μM PMSF (50 ml/g tissue) under cooling with ice (3×5 s) with an Ultraturrax homogenizer. After centrifugation ($3,000 \times g$, 5 min) the supernatant is transferred into new tubes and centrifuged ($200,000 \times g$, 1 h, 4°C). The pellet is washed with 2×5 ml of the same buffer, resuspended in 2 volumes of the same buffer and centrifuged again ($75,000 \times g$, 30 min). The pellet is resuspended again in the membrane buffer.

Membranes from Cultured β -Cells

Insulin producing cells from RINm5F cell culture (one culture flask) are washed twice with 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 0.5 mM EDTA, scraped with 20 ml of the same buffer and homogenized with 10 strokes of a tight fitting Potter-Elvehjem homogenizer followed by sonication (bath sonicator, 4°C , 10 s, maximal power). After centrifugation ($200,000 \times g$, 45 min, 4°C) the pellet is suspended in 10 ml of 25 mM HEPES/KOH (pH 7.4), 0.25 M sucrose, 100 mM NaCl, 0.5 mM EDTA, 200 μM PMSF and recentrifuged ($1,000 \times g$, 10 min). The supernatant is transferred to a new tube and centrifuged ($50,000 \times g$, 30 min, 4°C). The pellet is washed once with 25 mM HEPES/KOH (pH 7.4) and finally suspended in membrane buffer (25 mM HEPES/KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 μM PMSF, 10 $\mu\text{g}/\text{ml}$ soybean/trypsin inhibitor, 10 μM leupeptin, 1 mM iodoacetamide at 5 mg protein/ml). Aliquots are stored at -80°C .

K.6.6.12.2

Binding to Membranes

PURPOSE AND RATIONALE

Sulfonylureas block K_{ATP} channel in the β -cell plasma membrane (Schmid-Antomarchi et al. 1987a, b). Binding to the receptor and depolarization of the membrane initiates a chain of events leading to the release of insulin (Boyd 1992). The high affinity sulfonylurea receptor is considered to be an integral part of the K_{ATP} channel (Aguilar-Bryan et al. 1992). Binding

studies of sulfonylureas and other drugs can be performed with isolated pancreatic islets, isolated insulinoma cells, isolated intact membranes or solubilized membranes from primary or cultured cells or intact cells (Geisen et al. 1985, Gaines et al. 1988, Panten et al. 1989, 1992, 1993, Müller et al. 1994a).

PROCEDURE

Filter binding assays are performed in a total volume of 1 ml containing 5 mg membrane protein, 25 mM MOPS/KOH (pH 7.4), 0.1 mM CaCl_2 and labeled sulfonylurea, e. g. [^3H]glibenclamide or [^3H]glimipiride at concentrations between 0.1 and 20 nM. After incubation for 45 min at 25°C , the binding reaction is terminated by rapid filtration through Whatman GF/F filters soaked with the same buffer. The filters are washed three times with 5 ml of ice-cold 25 mM HEPES/KOH (pH 7.4), 100 mM NaCl, 1 mM EDTA, 200 μM PMSF, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 0.75 $\mu\text{g}/\text{ml}$ pepstatin and two times with 5 ml of ice-cold HEPES/KOH (pH 7.4), placed in 10 ml of ACSII scintillation cocktail and after incubation overnight counted for radioactivity in a liquid scintillation counter. Non-specific binding is determined in parallel samples in the presence of 1 μM unlabeled ligand.

K.6.6.12.3

Binding to Cells

Culture

Cultured cells producing the β -cell sulfonylurea receptor, SUR1, include the hamster insulin secreting tumor cell line, HIT-T15 (passage 65–75; CRL1777 ATCC). Cells are maintained in T-175 culture flasks as monolayers in DMEM containing high glucose supplemented with 10% fetal bovine serum, 100 U, 7 ml penicillin and 0.1 mg/ml streptomycin. Cells are grown in 5% CO_2 at 37°C , maintained in subconfluent cultures, fed three times a week, and subcultured as needed. To subculture, confluent cells are detached with 0.05% trypsin/EDTA, resuspended in supplemented DMEM/high glucose and replated at 1/10 of the original density.

Steady-State Analysis

After growth to 70% confluency, cells are treated with trypsin (0.02% trypsin in 0.9% NaCl/0.2 mM EDTA), washed with 3×10 ml KRBH containing 20 mM HEPES/KOH (pH 7.4), 125 mM NaCl, 5 mM KCl, 7.5 mM NaHClO_3 , 2 mM CaCl_2 , 0.8 mM MgSO_4 , suspended at a density of 2×10^7 cells/ml in the same buffer and then incubated for 45 min at 4°C at

a density of 4×10^6 cells per 0.5 ml assay volume with labeled sulfonylurea, e.g., [^3H]glibenclamide or [^3H]glimepiride at concentrations between 0.1 and 20 nM. For determination of non-specific binding, 1 μM unlabeled ligand is included. The incubation mixture is rapidly filtered on Whatman GF/C filters soaked with ice-cold buffer under reduced pressure. Filtration and washing has to take less than 30 s. The filters are washed three times with 6 ml ice-cold buffer containing 100 μM PMSF, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 0.75 mg/ml pepstatin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 50 mg/ml antipain dihydrochloride, placed in 10 ml of ACSII scintillation cocktail and after incubation overnight counted for radioactivity in a liquid scintillation counter.

Kinetic Analysis

For studying the association kinetics, the binding reaction is started by addition of radiolabeled ligand and terminated after predetermined periods of time by rapid filtration. For studying the dissociation kinetics, displacement of radiolabeled ligand at equilibrium (60 min incubation) is initiated by addition of unlabeled drug (final concentration 1 μM) and terminated after given periods of time. For termination, the incubation mixtures are rapidly chilled to 2–4°C by placing the assay tube in a dry ice/methanol bath for 1 s immediately prior to filtration in a filtration apparatus located in a cool bench. Further processing is performed as described above.

EVALUATION

K_d as well as B_{max} -values are calculated from Scatchard plot analyses and K_{on} and K_{off} values from kinetic studies. Curvilinear Scatchard plots allow the assumption of more than one binding site. IC_{50} values for half maximal inhibition of [^3H]sulfonylurea binding can be calculated from competition-inhibition plots.

MODIFICATIONS OF THE METHOD

Masuda and coworkers (1995) tested the effect of troglitazone on sulfonylurea receptor binding in rat pancreatic islet cells and in HIT cells. They concluded that troglitazone has a non-competitive binding site at, or in the vicinity of, the sulfonylurea receptor.

K.6.6.12.4

Photoaffinity Labeling of Membranes

PURPOSE AND RATIONALE

Binding sites of sulfonylureas have been identified in pancreatic β -cell membranes and in other tissues such

as brain. The technique of photoaffinity labeling allows the identification and purification of membrane receptors (Bernardi et al. 1988, Yip 1984, Kramer et al. 1994, Aguilar-Bryan et al. 1990, Boyd III 1991, Müller et al. 1994a, Geisen et al. 1985, Gaines et al. 1988, Kaubisch et al. 1982, Rajan et al. 1993, Sakura et al. 1995, Tanabe et al. 1998).

PROCEDURE

Photoaffinity Labeling with Glibenclamide

Samples of membrane suspension are prepared as described above. 600 μg of β -cell membranes, suspended in 100 mM sodium phosphate buffer (pH 7.4), are incubated in a total volume of 200 μl with 40–60 nM (0.3–0.4 μCi) [^3H]glibenclamide at 20°C in the dark for 60 min. Irradiation is performed in a Rayonet RPR 100 photochemical reactor (Southern Ultraviolet Co., CT), equipped with RPR 2530 Å lamps at a distance of 10 cm from the lamps. After irradiation at 254 nm for 2 min the membranes are diluted with 1 ml of 10 mM Tris/HEPES buffer (pH 7.4) containing 4 mM EDTA, 4 mM iodoacetamide, 4 mM PMSF and centrifuged (48,000 $\times g$, 30 min). The resulting pellet is resuspended in 200 μl water and protein precipitated according to Wessel and Flügge (1984).

Differential Photoaffinity Labeling

600 μg of β -cell membranes are incubated with various concentrations (10^{-9} to 10^{-4} M) of glibenclamide or the test compounds. After incubation with 60 nM (0.37 μCi) [^3H]glibenclamide in the dark, the membranes are photolabeled at 254 nm for 2 min. After washing of the membranes, the proteins are separated by SDS gel electrophoresis.

SDS-PAGE Analysis

The protein precipitates are dissolved in 70 μl of 62.5 mM Tris/HCl buffer (pH 6.8) containing 2% SDS, 5% mercaptoethanol, 0.005% bromophenol blue by shaking on a mixer for 60 min. After centrifugation (15,000 $\times g$, 10 min), the clear supernatants are subjected to SDS gel electrophoresis on 150 \times 180 \times 1.5 mm slab gels (Kramer et al. 1988). After fixing and staining, the gels are scanned with a densitometer (e.g. CD50, Desaga, Heidelberg). The radioactivity is determined by liquid scintillation counting after slicing the gels into 2 mm pieces and after digestion of proteins with Biolute.

EVALUATION

The radioactive peak of [^3H]glibenclamide bound to the receptor protein is decreased depending on the

concentration of unlabeled glibenclamide or test compounds. IC_{50} values can be calculated.

K.6.6.12.5

Binding to Recombinant SUR1

PROCEDURE

Heterologous Expression of SUR1

Chinese hamster ovary and COS (1, m6, 7) cell lines do not produce SUR1 and are used for heterologous SUR1 expression. For transient transfection, COS cells are plated at 50–60% confluence. Cells are transfected using either a DEAE-dextran or a lipofectamine protocol. 3-day-old cultures of COS cells are trypsinized and replated at a density of 3.5×10^5 cells per 35-mm well (six-well dish) and allowed to attach overnight. Typically, 5 μ g of a SUR1 plasmid is mixed with 5 μ g of a KIR6.2 plasmid and brought up to 7.5 μ l final volume in TBS (8 g/l NaCl, 0.4 g/l KCl, 0.2 g/l Na_2HPO_4 , 3 g/l Tris base, 0.2 g/l $CaCl_2$, 0.1 g/l $MgCl_2$, pH 7.5) before addition of DEAE-dextran (30 μ l of a 5 mg/ml solution in TBS). The samples are vortexed, collected by spinning in a microfuge, then incubated (15 min, 22°C) prior to addition of 0.5 ml of 10% FBS in TBS. Cells are washed twice with Hanks' balanced salt solution (HBSS), the DNA mix is added, and the cells are maintained in a CO_2 incubator at 37°C. After 4 h incubation, the DNA mix is decanted and the cells shocked for 5 min in 1 ml HBS and 10% dimethyl sulfoxide, then placed in 2 ml of DMEM/high glucose containing 2% FBS and 10 μ M chloroquine and kept in the incubator for 4 h. Thereafter the cells are washed three times with HBSS and incubated in normal growth media until assayed.

Alternatively, lipofectamine is used for transfection instead of DEAE-dextran. COS cells are plated in six-well dishes and are used at 70–80% confluency. Typically 1 μ g of a SUR1 plasmid and 1 μ g of a KIR6.2 plasmid are mixed with 375 μ l of Opti-Mem reduced serum medium (Life Technologies, Inc.), then added to 375 μ l of Opti-Mem containing 9 μ l of lipofectamine. After 1 h incubation, the mixture is supplemented with Opti-Mem to 1 ml final volume and added to the cells, which had been washed twice with 3 ml of Opti-Mem each. After 5 h incubation, the Opti-Mem is replaced with DMEM/high glucose containing 10% FBS. Transfections are scaled up based on the area of the plates used. For instance, 150-mm plates used for membrane isolations are transfected with 100 μ g of each plasmid using the DEAE-dextran protocol. Transfected cells are used for determination of sulfonylurea

binding and photolabeling or $^{86}Rb^+$ efflux 36–72 h post-transfection.

Membrane Preparation

Membranes are prepared from 15–25 150-mm dishes. Cells are washed three times in PBS (pH 7.4), then scraped in PBS and collected in 10-ml plastic tubes. The cells are pelleted, resuspended in 10 ml of hypotonic buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA), and allowed to swell for 1 h on ice. Cells are then homogenized, transferred to a 15-ml glass tube, and spun (2,000 $\times g$, 20 min, 4°C) to remove nuclei and unbroken cells. The supernatant is then transferred to a polycarbonate centrifuge tube and membranes are collected by centrifugation (50,000 rpm, 90 min, Ti80 fixed-angle rotor). The pelleted membranes are resuspended in 5–200 μ l of membrane buffer (25 mM Tris/HCl, pH 7.4, 2 mM EDTA, 250 mM sucrose, 0.2 mM PMSF, 10 μ g/ml leupeptin) and stored frozen in aliquots in liquid N_2 .

Membrane Solubilization

Membranes are rapidly thawed, resuspended using a Teflon-in-glass homogenizer, and mixed with ice-cold digitonin (25% w/v, in deionized water, prepared daily) to a final protein concentration of 3 mg/ml and 1% digitonin. Subsequent steps are performed at 25°C in the presence of 0.2 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml pepstatin, 0.2 mM phenanthroline. After 30 min of incubation, solubilized membrane proteins are separated from insoluble material by centrifugation (100,000 $\times g$, 1 h, 4°C). Alternatively, the thawed and re-homogenized membranes are mixed with the same volume (resulting in 3 mg/ml protein) of ice-cold 0.2% (w/v) phosphatidylcholine, 20% (w/v) glycerol, 280 mM KCl, 4 mM EDTA, 0.4 mM PMSF, 20 μ g/ml leupeptin, 50 mM HEPES/KOH (pH 7.4), 2.5% (w/v) Triton X-100 or CHAPS, incubated on ice for 1 h (under gentle stirring) and then centrifuged (150,000 $\times g$, 1 h, 4°C). The supernatants are collected and stored frozen in liquid N_2 .

Partial Purification of SUR1

For purification of the 140-kDa core glycosylated species of SUR1, 4-ml aliquots of digitonin-solubilized membranes are cycled for times over a 1-ml concanavalin A-Sepharose column equilibrated with 40 mM Tris/HCl (pH 7.5), 0.2 M NaCl, 1 mM EDTA, 1% digitonin. The column is washed with 8 ml of the equilibrating buffer and eluted with 4 ml of the equilibrating buffer containing 0.5 M methyl α -methylmannopyranoside. The eluted protein is stored

in liquid nitrogen. For purification of the 150-kDa complex glycosylated SUR1, wheat germ agglutinin-Sepharose is used instead of concanavalin A-Sepharose. The procedure is the same as described with the exception of elution of the receptor using 0.3 M N-acetylglucosamine.

The eluate for the lectin columns is cycled twice over a 1-ml column of Reactive Green 19-agarose equilibrated with 40 mM HEPES/KOH (pH 8.5), 1 mM EDTA, 0.5% (w/v) digitonin. After washing with 10 ml of equilibrating buffer, and 10 ml of the same buffer supplemented with 0.5 M NaCl, the protein is eluted with 4 ml of equilibrating buffer containing 1.5 M NaCl. The eluate from the Reactive Green-19 purification step is diluted 1:2 with 40 mM HEPES/KOH (pH 8.5), 1 mM EDTA, 0.2% digitonin, then cycled twice over a 1-ml phenylboronate-10 Sepharose column. The phenylboronate column is washed with 10 ml of the HEPES buffer, followed by 2 ml of 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, and 0.2% digitonin. The protein is eluted with 4 ml of 0.1 M Tris/HCl (pH 7.5), 1 mM EDTA, 0.15% (w/v) sodium dodecyl sulfate. Prior to binding measurements, pooled samples from the various column steps are concentrated by centrifugation ($3,000 \times g$, 30 min, 4°C) through 100,000 molecular weight cut-off filters (Amicon), which have been pretreated with 5% (v/v) Tween 20 for 14 h at 4°C.

K.6.6.12.6

Interaction with Extrapancreatic Tissues

In addition to β -cells and hypothalamic neurons (Angel and Bidet 1991), SUR1 is also expressed in human adipocytes (Shi et al. 1999). Closure of the K_{ATP} by sulfonylurea binding to the adipocyte SUR1 leads to depolarization of the plasma membrane, opening of voltage-dependent Ca^{2+} channels and concomitant increase of the cytosolic Ca^{2+} concentration. Thus, adipocytes and determination of intracellular Ca^{2+} may be used for assaying the potency of sulfonylureas with regard to binding to SUR1.

Prior to fluorometric Ca^{2+} measurement, the adipocytes are washed three times (by flotation) with HBSS (140 mM NaCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.9 mM NaH_2PO_4 , 4 mM $NaHCO_3$, 5 mM glucose, 2 mM sodium pyruvate, 2 mM glutamine, 20 mM HEPES, 1% BSA). The cells are then loaded with Fura-2-acetoxymethyl ester (10 μ M) in the same buffer for 45 min at 37°C in the dark with continuous shaking. For removal of extracellular dye, the cells are washed three times with HBSS and resuspended in

HBSS at 3.5×10^5 cells/ml. Cytosolic Ca^{2+} is determined using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. After establishment of a stable baseline in the absence or presence of the K_{ATP} channel openers, diazoxide and nitrendipine, the response to sulfonylureas is determined. Digitonin (25 μ M) and Tris/EGTA (100 mM) are used to measure maximal and minimal fluorescence to calibrate the signals. Cytosolic Ca^{2+} is calculated by the equation of Grynkiewicz and coworkers (1985).

Direct effects of sulfonylurea agents on glucose transport (Cooper et al. 1990, Rogers et al. 1987, Müller 2000), insulin-induced decrease in 5'-nucleotidase activity in skeletal muscle membranes (Klip et al. 1987), diacylglycerol-like activation of protein kinase C (Cooper et al. 1990) were studied in BC_3H_1 myocytes, rat cardiomyocytes (Bähr et al. 1995) and rat diaphragms (Müller et al. 1994b). Augmentation of the effects of insulin and insulin-like growth factors I and II on glucose uptake by sulfonylureas (Wang et al. 1987), coordinate regulation of glucose transporter function, and gene expression by insulin and sulfonylureas (Wang et al. 1989), glyburide-stimulated glucose transport *via* PKC-mediated pathway (Davidson et al. 1991) were studied in the same cell line. For glimepiride, a sulfonylurea of the 3rd generation, but not for sulfonylureas of the 2nd or 1st generation (glibenclamide or tolbutamide, respectively), its direct interaction with lipid rafts at the plasma membranes of rat adipocytes, presumably *via* spontaneous intercalation between raft glyco(sphingo)lipids has been reported (Müller and Geisen 1996, Müller and Welte 2002). This is associated with activation of the GPI-PL (see K.6.3.6.4) and the translocation of certain GPI-proteins and non-RTK from high cholesterol-containing rafts to low cholesterol-containing rafts accompanied by their activation. This redistribution of signaling components which ultimately leads to the IR-independent tyrosine phosphorylation of IRS-1 and downstream signaling to the glucose transport system in rat adipocytes (Müller et al. 2001, for reviews see Müller 2002, 2005) may represent the molecular basis for the so-called extrapancreatic activity of certain sulfonylurea drugs, i. e. the blood glucose lowering in the absence of additional insulin release. The effects of compounds/drug candidates on the redistribution and activation of lipid raft proteins in rat adipocytes can be assayed as described (see K.6.3.6.4.2).

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K.7

Measurement of Glucose Absorption

K.7.1

Inhibition of Polysaccharide-Degrading Enzymes

GENERAL CONSIDERATIONS

Starch as the predominant ingredient of human food is rapidly degraded in the gastrointestinal tract by salivary and pancreatic α -amylase to maltose which is further hydrolyzed by maltase localized in the brush border of the small intestine to glucose. Glucose is immediately absorbed leading to hyperglycemia and consequently to hyperinsulinemia. Both phenomena are undesirable in diabetics and in obese patients. Inhibition of the digestion of starch leads to a decrease and a retardation of glucose absorption. In nature, α -amylase inhibitors are found in wheat and other grains (Shaikin and Birk, 1970). Several inhibitors of amylase and α -glucosidase have been developed (Bischoff 1991). Animal experiments with high doses of absorbable α -glucosidase inhibitors indicate that lysosomal storage of glycogen may occur (Lembcke et al. 1991).

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K.7.1.1**Assay for α -Amylase****PURPOSE AND RATIONALE**

α -Amylase activity can be measured by determination of the reducing groups arising from hydrolysis of soluble starch by isolated pancreatic α -amylase according to the protocol of Rick and Stegbauer (1970). The reduction of 3,5-dinitrosalicylic acid to nitroaminosalicylic acid produces a color shift which is followed photometrically by changes in the absorbance at 546 nm. Inhibition of starch hydrolysis by an α -amylase inhibitor results in a diminished absorbance at 546 nm in comparison with the controls.

PROCEDURE

Commercially available pancreatic α -amylase (Merck, Germany) is used. Various concentrations of the α -amylase inhibitor are dissolved in 1 ml 20 mM Sørensen buffer, pH 6.9 and 10 mM NaCl. 0.1 ml pancreatic α -amylase solution in 0.4% BSA is added. After prior incubation at 25°C, the enzymatic reaction is started by addition of 1.0 ml soluble starch solution. The reaction is stopped after 10 min with 1.0 ml of dinitrosalicylic acid reagent. The mixture is heated in a boiling water bath for 10 min, and after cooling measured at 546 nm against the reagent blank.

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K.7.1.2**Assay for α -Glucosidase****PURPOSE AND RATIONALE**

Inhibition of glucosidase can be measured *in vitro* using glucosidase from porcine small intestinal mucosa.

PROCEDURE

Glucosidase is prepared from rat or porcine small intestinal mucosa or porcine pancreas. The enzyme activity is assayed according to Dahlqvist (1964). The inhibitory activity is determined by incubating a solution (20 μ l) of an enzyme preparation with 80 mM sodium phosphate buffer, pH 7.0 (500 μ l) containing 37 mM sucrose or maltose, or 3.7 mM isomaltose and a solution (20 ml) containing various concentrations of the inhibitor at 37°C for 20 min. The reaction mixture is heated for 2 min in a boiling water bath to stop the reaction. The amount of liberated glucose is measured by the glucose oxidase method. Matsuo and coworkers

(1992), Ikeda and coworkers (1991) and Glick and Bray (1983) studied the effect of intestinal disaccharidase inhibitors on obesity and diabetes.

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K.7.1.3**Everted Sac Technique for Assaying α -Glucosidase****PURPOSE AND RATIONALE**

The everted sac technique allows to study the effects of intestinal enzymes on substrates in an incubation vial.

PROCEDURE

Male rats weighing 120–140 g are sacrificed and the small intestines removed by cutting across the upper end of the duodenal junction. The intestine is stripped of the mesentery and the entire intestinal content is rinsed with cold saline solution. The intestine is divided into 7 to 8 cm-segments and turned inside out using a Pasteur pipette. The everted intestine is ligated at one end with a cotton thread and a second ligature is placed loosely around the opposite end ready for tying. A 1 ml syringe with Krebs-Henseleit-buffer is introduced into the lumen sac. The end of the sac is ligated and placed in a 25 ml Erlenmeyer flask containing 6 ml of 1% starch, dissolved in Krebs-Henseleit-buffer, with or without various concentrations of the α -amylase inhibitor. Pork α -amylase (4000 U/g starch) is also included in the 6 ml starch solution. Following gassing with 95% O₂/5% CO₂, the flask is tightly capped and incubated in a shaking bath at 37°C for 120 min. The reaction is terminated by the addition of 10 μ l 1 N HCl. At the end of the incubation period, the sac is removed from the flask and the inner fluid is collected by cutting one end of the sac. The final volumes of the solute in the serosal and the mucosal side and the level of glucose liberation are measured.

EVALUATION

Glucose liberated in the presence of various concentrations of the α -amylase inhibitor is expressed as percentage of glucose found without the inhibitor. Dose-

response curves can be drawn plotting percent inhibition versus concentration of the inhibitor

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K.7.2

Assays for GLUT2 Transport Activity

GENERAL CONSIDERATIONS

Diabetes management is directed toward control of postprandial fluctuations or excursions in blood glucose, which reflect the relative rates of delivery from the intestine and disposal to the tissues. While much is known about the impact of disposal mechanisms on blood glucose, rather less is known about delivery mechanisms, including gastric emptying, membrane hydrolysis, and sugar absorption itself.

Intestinal sugar absorption is increased in experimental diabetes (Csaky and Fischer 1981, Debnam et al. 1990, Thomson et al. 1981). Knowledge of sugar absorption has been based primarily on the mechanism of Na^+ /glucose cotransport by Na^+ /glucose cotransporter (SGLT1) and its long-term regulation by diet (Ferraris 2001). Recently, however, we have discovered a new pathway of sugar absorption, the apical GLUT2 pathway, which operates within minutes when high concentrations of primary digestion products, i. e., sugars, disaccharides, and α -dextrins, are presented to or generated at the apical membrane of the small intestine. This process is reversed when these sugars are absent from the lumen *in vivo*. The first indication of the existence of this mechanism was obtained in a study of the increase in fructose absorption in experimental diabetes (Corpe et al. 1996).

Dietary glucose crosses the apical membrane of the enterocyte by SGLT1 and exits across the basolateral membrane through the facilitative transporter GLUT2 (Thorens et al. 1990). Plasma glucose is maintained at ~ 5 mM. When the glucose concentration in the lumen is lower than in plasma, SGLT1 transports glucose uphill against its concentration gradient. This transcellular pathway is powered by a downhill gradient of Na^+ across the apical membrane, maintained by the basolateral Na^+/K^+ -ATPase. The cloning of SGLT1 by

Wright's laboratory (Hediger et al. 1987) and GLUT2 by Thorens' laboratory (Thorens et al. 1990) began the molecular biological era of intestinal nutrient transport

PURPOSE AND RATIONALE

Recent experimental evidence hints to a refined model for intestinal glucose absorption (for a review see Kellett and Brot-Laroche 2005, Kellett and Helliwell 2000) whereby a long-term diet containing high-glycemic index sugars is priming the rapid induction of the translocation to and insertion into the apical plasma membrane of GLUT2 by simple dietary sugars. Most importantly, the apical GLUT2 component of absorption seems to be several times greater than the active component at high glucose concentrations. According to this hypothesis, apical GLUT2 and SGLT1 act in tandem to cover the entire concentration range with SGLT1 playing an important regulatory role in the control of apical GLUT2. Apical GLUT2 is not only tightly regulated by long- and short-term supply of dietary sugars, but also by local and endocrine hormones, cellular energy status, stress, and diabetes; regulation occurs through a network of intracellular signaling pathways. Accordingly, apical GLUT2 can provide a major route of sugar absorption by which absorptive capacity is rapidly and precisely upregulated to match the dietary intake of sugars during assimilation of a meal (for a review see Kellett and Brot-Laroche 2005). Apical GLUT2 provides a safety factor by preventing high sugar loads from reaching the colon (Cheeseman 2002). Moreover, the consequent rapid delivery of sugar into the blood might increase the postprandial excursions, especially when glycemic control is poor. Apical GLUT2 is therefore a potential therapeutic target for novel dietary or pharmacological approaches to control intestinal sugar delivery and thereby improve glycemic control. The following assays enable the analysis of effects of compounds/drug candidates on the regulation (*via* insertion into the apical membrane) and transport activity of GLUT2 and may be helpful for the identification and characterization of a new class of glucose absorption inhibitors as putative future anti-diabetic drugs.

K.7.2.1

Perfusion of Jejunal Loops

PROCEDURE

Helliwell and coworkers (2000) described the perfusion of jejunal loops. Male Wistar rats (240–260 g) fed *ad libitum* on standard diet with free access to water are anaesthetized with an intraperitoneal injection

tion of a mixture of 1.0 ml of Hypnorm (Janssen U.K.) and 0.4 ml of Hypnovel (Roche Diagnostics) per kg of body weight. The intestine is exposed by a laparotomy. A 30-cm segment just below the ligament of Trietz is cannulated, flushed free of nutrients and perfused with a single-pass system at a flow rate of 0.5 ml/min with a modified Krebs bicarbonate saline (118 mM NaCl, 4.74 mM KCl, 1.2 mM KH_2PO_4 , 1.25 mM CaCl_2 and 24.8 mM NaHCO_3) segmented with 95% O_2 /5% CO_2 . The effluent is collected every 5-min using a fraction collector. The jugular vein is cannulated with a silastic tube and infused with saline at a flow rate of 3 ml/h. Radiolabeled substrate is added to the perfusate and radiolabeled PEG4000 is included as a non-absorbable marker to allow for the correction of fluid movements. At the end of the perfusion period the intestine is removed, weighed wet and then dried overnight before reweighing. For the addition of phloretin the luminal solution required the use of modified Krebs bicarbonate saline (see above) to keep the inhibitor in solution. Phloretin is initially made up as a stock solution in DMSO and 200 μl of stock, then added to 150 ml of perfusate to give a final concentration of 1 mM. Final DMSO concentration is therefore 0.13% (v/v), which has been demonstrated previously to have no effect on tissue permeability. Phloretin can be used in solution for the length of the experiment.

Compound Application

Because compounds take some time to become effective in whole tissue and because there is a limit to the time of viability for isolated loops *in vitro*, the following procedure for the treatment of jejunum with drugs has been adopted. Jejunum is first perfused luminally *in vivo* with 5 mM D-fructose (plus 1 mM β -hydroxybutyrate as an energy source) in the presence or absence of compound. The perfusion system is a gas-segmented, single-pass system with perfusate and gas flow rates of 0.75 and 0.38 ml/min respectively. Jejunum is perfused with compound for 30 min. At the end of the *in vivo* treatment period, the cannulated loop is then excised and perfused *in vitro* using the gas-segmented, recirculated flow system: 5 mM D-glucose or fructose in combination with the compound at the end of the *in vivo* treatment period are perfused luminally through the jejunum for 60 min; the flow rates of perfusate and gas were 7.0 and 3.4 ml/min respectively. Control perfusions are performed in which no compounds are present. These show that there is no falling off in the glucose/fructose uptake rate after the initial approach to the steady state, confirming that this approach to compound treatment and perfu-

sion ensured that the jejunum is viable for the whole experimental period from the start of the *in vivo* to the finish of the *in vitro* perfusion. Samples (0.05 ml) are taken from the perfusate at 5-min intervals throughout the *in vitro* perfusion. After measurement of glucose/fructose concentration with a COBAS automatic analyser (Roche Diagnostics) using a test kit from the same supplier, the amount of glucose/fructose remaining in the perfusate is calculated, with correction for losses in perfusate volume caused by water transport. Because the perfusate is recirculated, the concentration of fructose decreases with time; it is therefore necessary to limit the period over which the rate of transport is measured to one in which the concentration decreased by no more than 20%. Over such periods, plots of luminal perfusate content versus time are effectively linear. The rate of glucose/fructose transport, expressed as $\mu\text{mol}/\text{min} \cdot (\text{g of dry weight})^{-1}$, is therefore determined by linear regression analysis as the average rate of disappearance over this period from the luminal perfusate *in vitro*.

Dually Perfused *In Situ* Jejunum

Dual perfusion of the jejunum is typically performed as described by Hirsh and Cheeseman (1998). Male Sprague-Dawley rats (200–350 g) are fed a standard chow diet and water *ad libitum*. Before the start of the experiment, food is withdrawn for approximately 24 h to minimize intestinal luminal contents during surgery. All rats are anesthetized prior to surgery using sodium pentobarbital given by intraperitoneal injection (60 mg/kg body weight) and placed on a heated (37°C) surgical table. After performing a laparotomy, the blood supply to the spleen, rectum, colon, cecum, stomach, and ileum are tied off and the tissues removed. The vasculature to the pancreas and duodenum are also ligated. A 35-cm segment of jejunum, starting 5 cm distal from the ligament of Trietz, is isolated and the luminal contents removed by gently flushing with 20 ml of warm saline (0.9%), and the jejunum is cannulated at both ends. The lumen is perfused with a Krebs-bicarbonate saline solution (120 mM NaCl, 4 mM KCl, 2.5 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , 1 mM CaCl_2) using a pump. The solution containing 5 mM 3-*O*-MG or 5 mM 3-*O*-MG and 5 mM D-fructose, is maintained at 37°C and gassed with 95% O_2 , 5% CO_2 . Isotopically labeled hexoses, 10 μCi of either 3-*O*-methyl-D-[1- ^3H]glucose, or both 3-*O*-methyl-D-[1- ^3H]glucose and D-[U- ^{14}C]fructose, are added to the luminal circuit immediately after portal vein cannulation. The single-pass luminal circuit is perfused at a flow rate of 1.6 ml/min, and the so-

lution is segmented by 95% O₂/5% CO₂ gas bubbles. The gas bubbles are introduced into the luminal perfusate through a Y piece at a flow rate which ensured that bubbles occupied the diameter of the perfusion tube. This not only exposes the tissue to a saturating gas partial pressure, but also helps to mix the solution in the tissue lumen. After a single-pass through the segment of jejunum the luminal perfusate is discarded. The aorta, proximal to the superior mesenteric artery, is ligated just prior to insertion of a cannula into the superior mesenteric artery. The single-pass vascular circuit is perfused at a rate of 1.6 ml/min with fresh Krebs-bicarbonate saline solution, containing 5 mM D-glucose, 0.034 mM streptomycin sulfate, 5 mM L-glutamine, 1120 USP units heparin, and 10% w/v Ficoll 70 as a plasma expander, which is maintained at 37°C and gassed with 95% O₂/5% CO₂ maintaining the pH at 7.4. Once the vascular circuit is established, the rat is euthanized and the vascular perfusate is collected *via* cannula placed in the hepatic portal vein. The effluent is collected continuously for up to 80 min using a fraction collector.

Washout Studies in the Dually Perfused Jejunum

This procedure is similar to the one used by Boyd and Parsons (1979). This washout model is useful for indirectly indicating the locus of compound/drug action. When the labeled 3-*O*-MG is washed out in the presence of an equimolar concentration of unlabeled mannitol in the lumen, the rate of washout into the vascular bed can be described by the sum of two exponential terms (assuming a two-compartmental model applies). The contributing compartments are a fast releasing (vascular flow rate dependent) and a slow releasing one (vascular flow rate independent), which represent: 1) Q_{01} mucosal epithelium layer and 2) Q_{02} deeper submucosal (muscle) layer. Assuming that each pool unloads independently, then each pool will have its own rate constant: the fast, K_1 and the slow, K_2 .

EVALUATION

Tissue ion conductance (G) is calculated from PD and I_{sc} according to Ohm's law. For the measurement of basal glucose fluxes, the tissue is clamped at zero voltage by continuously introducing an appropriate I_{sc} with an automatic voltage clamp every 5 min, except for 5–10 s when PD is measured by removing the voltage clamp. Tissue pairs are matched for conductance and discarded if conductance varied by >20%. The non-metabolizable glucose analog 3-*O*-methyl-D-[1-³H]glucose (5 μ Ci) is added either to the serosal or mucosal side after mounting and the tissue is allowed

to equilibrate for 20 min. Net directional flux from mucosal-to-serosal surface is determined for conductance-matched tissues by measuring four consecutive 5-min fluxes before the addition of compound/drug candidate and four 5-min fluxes following the addition of the agent.

MODIFICATION OF THE METHOD

Epithelial glucose uptake can be measured according to the method described by Walker and coworkers (2005).

K.7.2.2

Transport Activity of Brush Border Membrane Vesicles

Preparation of Intestinal Brush Border Membrane Vesicles

Brush-border membrane vesicles (BBMV) are prepared from mucosal scrapings as described by Corpe and coworkers (1996) on basis of a standard procedure introduced by Maenz and Cheeseman (1986). Every stage of the preparation is performed at 0–4°C to prevent changes in trafficking after the intestine had been excised. Briefly, the jejuna of two rats are perfused with compounds/drugs as described above. The timing of the final *in vitro* perfusion is such as to get as direct a correspondence as possible between rates of transport and extent of trafficking. Perfusions for vesicle preparations are therefore terminated at a point corresponding to half the time period over which the average rate of transport is determined, as described above. Immediately after perfusion, each jejunum is rinsed with ice-cold buffered mannitol (20 mM imidazole buffer, pH 7.5, containing 250 mM mannitol and 0.1 mM PMSF), placed on an ice-cold glass plate and slit longitudinally so that the muscle of the jejunum flattened out on to the cold plate. The mucosa is scraped off with a microscope slide and placed in 65 ml of ice-cold mannitol/Tris buffer (300 mM mannitol, 5 mM EGTA, 12 mM Tris/HCl, pH 7.4, and 0.1 mM PMSF). The tissue is homogenized in a Polytron homogenizer (four 30 s bursts using the large probe at setting 7) before addition of MgCl₂ to a final concentration of 12 mM. After stirring the solution on ice for 15 min the solution is centrifuged (1,600 \times g, 15 min) to remove debris. The supernatant is further centrifuged (20,000 \times g, 30 min) and the pellet homogenized in half-strength mannitol/Tris buffer (150 mM mannitol, 2.5 mM EGTA, 6 mM Tris/HCl, pH 7.4, and 0.05 mM PMSF) with a glass homogenizer before further addition of MgCl₂. After stirring on ice the centrifugation is repeated as before, and the pellet is then washed

(300 mM mannitol/5 mM Tris/HCl, pH 7.4) before re-pelleting ($20,000\times g$). This BBMV preparation is diluted to a protein concentration of 8 mg/ml. Enrichment of sucrase activity in these highly purified preparations typically range from 16- to 20-fold. There is no significant enrichment of Na^+/K^+ -ATPase activity.

Preparation of Renal Brush Border Membrane Vesicles

Both kidneys are removed from anaesthetized animals and placed in ice-cold 154 mM NaCl. After removal of the renal capsule, the kidneys are weighed and then sliced into 2-mm thick sections and the cortex dissected away. Cortical fragments from six kidneys are pooled, weighed and suspended in 30 ml of 300 mM mannitol, 12 mM Tris/HCl (pH 7.4), 5 mM EGTA. The resulting suspension was homogenized for 2 min (Ultra Turrax homogenizer) at half speed, followed by the addition of 42 ml of cold distilled water and MgCl_2 to a concentration of 12 mM, and then stirred on ice for 15 min. The solution is then centrifuged ($2,000\times g$, 15 min) and the supernatant then re-centrifuged ($21,000\times g$, 30 min). The pellet is suspended in 20 ml of 150 mM mannitol, 6 mM Tris/HCl (pH 7.4), 2.5 mM EGTA using 10 cycles of a hand-operated glass-Teflon homogenizer. MgCl_2 is added to a concentration of 12 mM and, after stirring on ice for 15 min, low and high-speed centrifugations described above are repeated. The pellet is then re-suspended in 20 ml of 300 mM mannitol, 12 mM Tris/HCl (pH 7.4), 2.5 mM EGTA and centrifuged ($21,000\times g$, 30 min). The purified BBMV pellet is finally resuspended in the same buffer to a protein concentration of 3–6 mg/ml using 5–6 passes through a syringe fitted with a 21-gauge needle. All steps are carried out at 4°C. The concentration of protein (Bradford 1976) and activity of alkaline phosphatase and Na^+/K^+ -ATPase in the initial homogenate and BBMV preparation are determined in order to derive values for enrichment of these marker enzymes (see Corpe et al. 1996).

Glucose Transport

Transport studies are carried out at 20°C on freshly prepared BBMV as described (Sharp and Debnam, 1994). The transport process is initiated by mixing equal volumes of BBMV suspension and uptake buffer consisting of 200 mM NaSCN, 20 mM HEPES, 0.1 mM MgSO_4 containing D- ^3H glucose (9 Ci/mmol) and such that the final concentration of glucose is 5–500 μM . Due to the wide glucose concentration range used, the osmotic balance is maintained by adding mannitol to give a final glucose plus mannitol concentration of 100 mM. Uptake is terminated af-

ter 4 s by the addition of 3 ml of ice-cold 154 mM NaCl containing 0.5 mM phlorizin, followed by vacuum filtration through 0.45- μm nitrocellulose filters (Sartorius, Germany). Three further washes with stop solution are carried out.

EVALUATION

Scintillation counting of the filters is used to calculate glucose accumulation and this is expressed as $\text{nmol}(\text{mg protein})^{-1}(4\text{ s})^{-1}$. The kinetic parameters of V_{max} (maximum transport capacity) and K_t (glucose concentration at half V_{max}) for phlorizin-sensitive uptake were derived using a non-linear least squares curve-fitting program.

Parallel uptakes using buffer containing 1 mM phlorizin to block SGLT-mediated transport indicated that uptake is >90% phlorizin-sensitive. In order to assess GLUT-mediated transport, a higher glucose concentration (20 mM) is used in the presence of 1 mM phlorizin. This value is consistent with the low affinity of GLUT transporters for glucose binding. In addition, uptake of L-glucose at both 100 μM and 20 mM should be performed to establish the contribution of passive glucose transport.

K.7.2.3

Apical Expression of GLUT2

Biotinylation of Surface Proteins

The method of surface protein biotinylation for analysis of GLUT2 expression at apical membranes has been introduced by Au and coworkers (2002). For the detection of GLUT2 in the apical membrane of enterocytes, i.e. of the translocation of GLUT2 from intracellular vesicles to the apical cell surface, proteins expressed on the apical surface of jejunal enterocytes are labeled with *N*-hydroxysuccinimido (NHS)-SS-biotin introduced into the intestinal lumen. At the end of the *in vivo* perfusion the intestine is maintained *in situ*, but cooled on ice. The luminal solution containing NHS-SS-biotin, 1.5 mg/ml in 10 mM triethanolamine/2.5 mM CaCl_2 /250 mM sucrose buffer (pH 9.0), is introduced into the lumen and left for 30 min. The lumen is then flushed with a PBS/100 mM glycine buffer to quench the free biotin before two final washings with PBS. Mucosal scrapings are then used to make protein extracts as described below.

Isolation of Biotinylated Proteins

Proteins are extracted from the homogenate for 1 h at 4°C using the following buffer: 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 50 mM Tris (pH 7.5).

After centrifugation ($14,000\times g$, 10 min) the supernatant is collected and incubated overnight with streptavidin beads. After washing twice with the Triton X-100 buffer to remove non-linked protein, the beads are washed with a high-salt buffer (500 mM NaCl) and finally with a no-salt buffer (10 mM Tris, pH 7.5). The isolated biotinylated proteins are then solubilized in Laemmli sample buffer to be run on SDS-PAGE for immunoblotting. Comparisons of total cell GLUT2 with apical GLUT2 are made by running samples of the supernatant after spinning down the streptavidin-coated beads on the same immunoblots as samples of recovered biotinylated protein.

Detection of Apical GLUT2 by Immunoblotting

Membranes (15 μ g of protein) are solubilized in Laemmli sample buffer and separated by SDS-PAGE (10% gel). The proteins are blotted on to nitrocellulose membrane by electrotransfer for 75 min at 4°C. The membranes are stained for total protein with Ponceau S to ascertain that equivalent amounts of protein are loaded and transferred from each lane. Blocking of the membrane is carried out in 3% non-fat milk in PBST (0.05% Tween 20/PBS, pH 7.4) for 1 h and then incubated with 1:1,000 rabbit polyclonal antibody to rat GLUT2 in 3% non-fat dry milk in PBST overnight at 4°C. The membrane is washed three times in 3% non-fat dry milk/PBST for 15 min, 1 h and 15 min respectively. The nitrocellulose membrane was then incubated with a secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase diluted 1:2,000 in 3% non-fat dry milk/PBST for 1 h. Three subsequent washes followed as described above. Finally, the membrane is treated with the ECL detection solution (Amersham-Pharmacia) before autoradiography for 30 s using Kodak XAR-5 film with an intensifying screen. The specificity of the antibody was determined by running parallel lanes of isolated biotinylated proteins and probing them with the native antibody or with antiserum which had been pre-incubated at room temperature for 1 h with the peptide used to raise the antibody. Two antibodies are employed, one that recognizes an epitope in the large extracellular loop between transmembrane segments 1 and 2, amino acids 40–55 (SHYRHVLGVPLDDRRRA; Biogenesis, UK) and a second which recognized a portion of the C-terminal sequence of rat GLUT2 (CVQMEFLGS-SETV; Research Diagnostics, USA).

K.7.3

Evaluation of Glucose Absorption In Vivo

PURPOSE AND RATIONALE

The inhibition of glucose absorption can be determined by measuring blood glucose after administration of starch or disaccharides with and without the inhibitor. In addition, non-absorbed starch or disaccharides can be determined in the intestine.

PROCEDURE

Male Wistar rats are kept on a standard diet with free access to tap water at constant temperature ($24 \pm 1^\circ\text{C}$). Sixteen hours prior to the experiment food but not water is withheld. Groups of rats receive by stomach tube 2.5 g/kg raw starch in a water suspension without or with various doses of the α -amylase inhibitor. After 10, 20, 30, 60, 120 and 240 min, blood is withdrawn for determination of blood glucose and non-esterified fatty acids. The animals are sacrificed after these intervals and the residual starch in the stomach and the intestine determined. Definitely more starch is found in the intestine after simultaneous application of the α -amylase inhibitor. Similar experiments are performed in dogs for determination of serum insulin. The increase of blood glucose and serum insulin as well as the decrease of NEFA are inhibited.

EVALUATION

The values of starch content in stomach and intestine, as well as the blood glucose-, serum insulin- and NEFA-values are compared between control and treated animals.

MODIFICATIONS OF THE METHOD

In order to test the inhibition of glucosidase in addition, loading tests with sucrose or other disaccharides are performed (Puls and Keup 1973, Puls et al. 1977, Matsuo et al. 1992). Matsuo and coworkers (1992) performed experiments with genetically or experimentally obese rats (female Zucker fatty rats or female ventromedial hypothalamic nuclei-lesioned rats) and male yellow *KK*-mice. LeMarchand-Brustel and coworkers (1990) studied the effect of an α -glycosidase inhibitor on experimentally induced obesity in mice. Male Swiss albino mice were rendered obese by injection of goldthioglucose at the age of 3 weeks. Since goldthioglucose treatment does not produce obesity in all injected mice, pro-obese mice were selected at 8 weeks of age on the basis of their body weight gain. They were divided in a control group receiving chow without drug and a treated group receiv-

ing chow containing the glycosidase inhibitor. Weight gain, glycemia and insulinemia were followed over 120 days. Madar and Omusky (1991) studied the inhibition of intestinal α -glucosidase activity and postprandial hyperglycemia by α -glucosidase inhibitors in *fa/fa* rats. Various starch sources were used. Blood samples were taken at various intervals after starch load. Takami and coworkers (1991) studied the antidiabetic actions of a disaccharidase inhibitor in spontaneously diabetic (GK) rats. Okada and coworkers (1992) reported anti-obesity and antidiabetic actions of a new potent disaccharidase inhibitor in genetically obese-diabetic mice.

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K.8

Monitoring of Diabetic Late Complications

K.8.1

Aldose Reductase Activity

GENERAL CONSIDERATIONS

Secondary symptoms of long lasting diabetes mellitus are diabetic neuropathy with sensory symptoms, motoric disturbances due to reduced nerve conduction velocity and diabetic cataracts. Both are related to enhanced conversion of glucose to polyols, such as sorbitol, by the enzyme aldose reductase (van Heyningen 1959, Clements 1979). Sorbitol is converted to fructose by sorbitol-dehydrogenase. A low activity of this enzyme enhances the accumulation of sorbitol, thus contributing to cellular damage. Inhibitors of aldose reductase have been developed with positive results in diabetic patients (Kador et al. 1985).

There are even some experimental hints for the benefit of aldose reductase inhibitors in diabetic cardiomyopathy (Cameron et al. 1989). Experimental data provide also evidence for possible beneficial effects in diabetic neuropathy, retinopathy, measured by nerve conduction velocity or electroretinography, lens cataract formation, vascular permeability and filtration (Williamson et al. 1987, Tilton et al. 1988 and 1989, Pugliese et al. 1990) and nephropathy (Sarges and Oates 1993).

Geisen and coworkers (1994) reported on sorbitol-accumulating pyrimidine derivatives which inhibited sorbitol dehydrogenase, induced a dose-dependent increase of tissue sorbitol and accelerated cataract development. Surprisingly, an acceleration of motor nerve conduction velocity in normal and diabetic rats and a normalization of glomerular filtration rates in diabetic rats were found.

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Using this preparation, the *in vitro* effectiveness of various aldose reductase inhibitors can be determined (Varma and Kinoshita 1976, Billon et al. 1990).

PROCEDURE

Aldose reductase from lenses of calf eyes is isolated from the homogenates by ammonium sulfate precipitation of the concomitant proteins and by column chromatography on DEAE-cellulose. DL-Glyceraldehyde is used as substrate. The activity is expressed as the rate of OD_{340} [nm] due to the utilization of NADPH in the reaction. The reaction mixture contains 0.1 M phosphate buffer (pH 6.2), NADPH 2.5×10^{-4} M, DL-glyceraldehyde 1.5×10^{-3} M and the enzyme. The total volume of the reaction mixture is 1 ml. The reference blank consists of all the above compounds except the substrate. The effects of inhibitors on the enzyme activity are determined by including the compound being tested at the desired concentration in the reaction mixture. Appropriate blanks are used to correct for nonspecific reduction of NADPH and absorption by the compounds being tested. The test compounds are added first in 10^{-3} M solution and diluted as desired.

EVALUATION

Percentage of inhibition is tested at various concentrations and IC_{50} -values are calculated.

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K.8.1.2

Measurement with Cataract Lenses

PURPOSE AND RATIONALE

The effect of aldose reductase inhibitors can be tested by determining the level of aldose reductase, NADPH and $NADP^+$ in the lenses of galactose treated or streptozotocin diabetic rats after treatment with the inhibitor compared to controls.

PROCEDURE

Galactose Treatment

Cataract formation can be induced by galactose feeding to young rats. Male Sprague-Dawley rats weighing 40–50 g are randomly divided into 2 groups. One

K.8.1.1

Measurement with Normal Lenses

PURPOSE AND RATIONALE

The enzyme aldose reductase from bovine lens has been characterized by Hayman and Kinoshita (1965).

group is fed a laboratory chow, the other group is fed a galactose diet containing 50% galactose, 20% cornstarch, 15% casein, 9% hydrogenated oil, 4% salt mixture, and 2% cod liver oil. Diabetes is induced in male Sprague-Dawley rats weighing 80–90 g by a single intravenous dose of 100 mg/kg streptozotocin.

Streptozotocin Treatment

Diabetes is induced in 230–260 g male Sprague-Dawley rats by administration of a single tail vein injection of streptozotocin (55 mg kg^{-1}) 2–4 weeks prior to study. Streptozotocin is dissolved in freshly prepared 0.05 M citrate buffer (pH 4.5) and administered under light isoflurane anaesthesia. Animals are glycosuric 24 h after streptozotocin treatment. The weight of the control animals is matched to that of the 2–4 week diabetics. Animals were allowed *ad libitum* access to a standard rat chow (Diet RM1, SDS Ltd, UK) and water until the time of experimentation, with the exception of those subjected to an overnight fast. For all experimental procedures animals are terminally anaesthetized with intraperitoneal pentobarbitone sodium (90 mg kg^{-1}) before removal of eyes. The progression of cataracts is observed in controls and in rats treated with the aldose reductase inhibitor.

Assay

At appropriate times, the rats are sacrificed and the lenses are dissected. They are frozen on solid CO_2 and stored at -70°C until analysis. Single rat lenses are homogenized in 200 μl of 10 mM sodium phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0) for 0.5 min at 0°C . A cell-free extract is obtained by centrifuging ($17,300 \times g$, 10 min) the total lens homogenate. The enzyme reaction mixture (final volume 1 ml) contains 0.4 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 M HEPES/NaOH buffer (pH 7.0), 10 mM DL-glyceraldehyde and 0.12 mM NADPH. A 20- μl aliquot of supernatant is added to initiate the reaction. Decrease in absorbance at 340 nm is followed spectrophotometrically. Enzymatic activity is expressed as nM of NADPH oxidized per min.

EVALUATION

Results are expressed as aldose reductase units/mg lens (wet weight). Means \pm SE are calculated and compared using Student's non-paired *t*-test.

MODIFICATIONS OF THE METHOD

Naeser and coworkers (1988) studied sorbitol metabolism in the retina, optic nerve, and sural nerve

of diabetic rats treated with an aldose reductase inhibitor. Activities of aldose reductase, sorbitol-dehydrogenase, and content of sorbitol were assayed in these tissues.

Freeze-dried tissue samples are sonicated for 5–10 s in 215 μl of 0.04 M Tris (pH 6.8). For determination of aldose reductase, the homogenate is centrifuged for 45 min at 37,000 rpm and 100 μl is taken from the supernatant for analysis. The remaining sample is centrifuged further (96,000 rpm, 2 h) and the supernatant used for sorbitol determination. For determination of aldose reductase, 10 μl of the sample are mixed with 15 μl buffer solution containing 0.14 mM Tris, 151 μM NADH, and 0.6 M glucose. After incubation in a water bath for 20 min at 38°C , the incubation is stopped by transfer to ice. Samples mixed with buffer solution without glucose serve as blanks. Twenty μl of the incubated material are then mixed with 1.25 ml of 0.04 M NaOH solution containing 1.7 mM NaCl. Fluorescence is measured in a Farrand Ratio Fluorometer with primary filters 5860 and 5970 (Corning Glass Works) and secondary filters 2A (Turner Optical Co), 4308 and 3387 (Corning Glass Works). The amount of NADPH consumed serves as measure for aldose reductase activity.

Sorbitol-dehydrogenase is measured by adding 5 μl of fresh homogenate to 20 μl of a buffer composed of 0.1 mM Tris (pH 6.8), 1.25 mM NAD and 50 mM sorbitol. After incubation for 30 min at 38°C , the reaction is stopped by the addition of 5 μl 0.1 N NaOH and transfer to ice. The amount of NAD^+ converted to NADH is determined luminometrically in 10 μl samples with 100 μl of NAD(P)H reagent (Roche Diagnostics) containing 5 mg/L luciferase, 25 mM potassium phosphate, 100 mM dithiothreitol, 38 U/L Triton-X 100, and 20 mM myristic aldehyde. The flux of light is instantly registered, using a photomultiplier.

Sorbitol is determined in the centrifugation supernatant. After immersion in boiling water for 5 min, aliquots of 15 μl are added to 10 μl buffer containing 0.1 M Tris (pH 8.6), 3.5 mM NAD, and 2.7 U/ml sorbitol-dehydrogenase. Tissue blanks are run without sorbitol-dehydrogenase. The incubation is terminated and the amount of NADH formed is determined as for the sorbitol-dehydrogenase assay.

Gonzales and coworkers (1986) studied the effect of an aldose reductase inhibitor on integration of polyol pathway, pentose phosphate pathway and glycolytic route in diabetic rat lens. Diabetes was induced in male Wistar rats weighing about 230 g by subcutaneous injection of 200 mg/kg alloxan monohydrate. After one week, in the diabetic rat lens was an ap-

parent increase in the flux of glucose through the pentose phosphate pathway, as measured by the difference in the yields of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ [C1–C6]. Treatment with the aldose reductase inhibitor reduced the values toward normal. With glucose tritiated on carbons 2 or 3, it has been shown that the flux of glucose through the polyol route is increased, whereas the flux through the glycolytic pathway is decreased in the diabetic rat lens. Both parameters were restored to normal in diabetic rats treated with the aldose reductase inhibitor.

Meydani and coworkers (1994) investigated the onset and progression of cataract in weanling Sprague Dawley rats fed 10, 15, 20 and 30% dietary galactose for 45–226 days.

Ohta and coworkers (1999) studied cataract development in 12-months-old rats fed a 25% galactose diet and its relation to osmotic stress and oxidative damage.

Using phosphorus-31 nuclear magnetic resonance spectroscopy, Sakagami and coworkers (1999) investigated the metabolic kinetics of organophosphate compounds in the rat lens during cataract development induced by different doses of galactose added to rat chow.

Mackic and coworkers (1994) developed a model of galactose-induced cataract formation in guinea pigs and studied the morphologic changes and accumulation of galactitol.

Sato and coworkers (1998) demonstrated that the formation and progression sugar cataracts in galactose-fed dogs can be dose-dependently inhibited by the aldose reductase inhibitors.

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K.8.2 Nerve Conduction Velocity

PURPOSE AND RATIONALE

Aldose reductase inhibitors can be tested in rats with diabetes induced with streptozotocin by measuring nerve conduction velocity (Miyoshi and Goto 1973, Mayer and Tomlinson 1983, Tomlinson et al. 1882, 1984, Yue et al. 1982) and resistance to ischemic conduction block (Price et al. 1988).

PROCEDURE

Male Wistar rats weighing 100–150 g are rendered diabetic with an i.v. injection of 60 mg/kg streptozotocin. Diabetes is confirmed by the presence of glucosuria and elevated blood glucose levels 24 h after injection. The rats are kept on standard diet and water ad libitum for 28 days after streptozotocin treatment. Groups of rats are treated with the test compound (aldose reductase inhibitor) or the vehicle once daily by gavage starting 24 h after streptozotocin injection. One group of age-matched rats serves as control.

For measurement of sciatic nerve conduction velocity, rats are anesthetized with 2% halothane in oxygen and placed in a Perspex chamber that is maintained at a constant temperature by a copper coil in the base through which warm water is circulated. A fine needle thermocouple is inserted in the lateral aspect of the left hind limb, and the subcutaneous temperature is measured with an electronic thermometer. The conduction velocity of the left sciatic-tibial nerve is measured by a modification of the method of Sharma and Thomas (1974). Two fine platinum stimulating electrodes are inserted in the sciatic notch, and two more are inserted through the skin of the ankle to lie adjacently to the tibial nerve. The electrodes are 5 mm apart, the distal end being the cathode. A recording electrode is inserted under the skin of the lateral side of the foot and another between the third and fourth toes. A ground electrode is inserted under the skin on the dorsum of the foot. Both stimulation and recording are carried out with a neurophysiological unit (e.g., model MS92a, Medelec, Old Woking, UK). The nerve is stimulated supramaximally with square-wave pulses of 0.1 ms duration. The conduction velocity is calculated as the distance between the distal electrodes divided by the difference between the latencies at the two stimulation points. The mean of three recordings

is taken. The coefficient of variation of nerve conduction velocity is calculated by measuring nerve conduction velocity six times in one rat from each treatment group, withdrawing and reinserting the electrodes each time.

To measure resistance to ischemic conduction block, two fine platinum stimulating electrodes (1 cm apart) are inserted in the lateral aspect of the tail 3 cm from the base. Two recording electrodes are inserted near the tip, and a ground electrode is inserted midway between these and the stimulating electrodes. A thermocouple is attached to the middle of the tail. Both stimulation and recording are carried out as described above. To render the tail ischemic, a small sphygmomanometer cuff is inflated to 240 mm Hg around the base of the tail. The nerve is stimulated at 2.5 min intervals until the action potential disappears. The tail temperature is maintained at 32°C. At the end of the experiment, 5 ml blood is taken by cardiac puncture. The animal is then sacrificed, the sciatic nerves are removed, cleaned, weighed, snap-frozen in liquid nitrogen and stored at -20°C for subsequent estimation of polyol level.

Nerve polyol estimation is performed by the method of Stribling and coworkers (1985). Frozen nerves are thawed, placed in 1 ml water containing 60 µg of α -methylmannoside as an internal standard, and boiled for 20 min. Zinc sulfate (200 µl of a 5% solution) is added, samples are homogenized with a Polytron homogenizer, 200 µl 0.3 N barium hydroxide solution is added, and the samples rehomogenized. After centrifugation, the supernatant is freeze-dried overnight. The residue is silylated with 250 µl of a mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane (10/2/1, Pierce) at room temperature for 24 h. The reaction is stopped by adding 2 ml water, and 200 µl cyclohexane is added. Samples are shaken and 2 µl of the organic phase is injected into a gas chromatograph. Plasma glucose levels are measured with the use of a glucose analyzer. Plasma fructosamine, which reflects plasma protein glycosylation, is measured with the use of a commercial kit. Plasma glucose, plasma fructosamine and nerve sorbitol are increased and nerve conduction velocity decreased in streptozotocin treated rats.

EVALUATION

Data from diabetic rats treated with the aldose-reductase inhibitor are compared with the values of untreated diabetic rats and normal controls. Statistical comparisons are made by use of the Mann-Whitney *U* test.

MODIFICATIONS OF THE METHOD

Sima and coworkers (1990) studied motor nerve conduction velocity and neuroanatomical abnormalities in insulin-deficient diabetic Bio-Breeding rats (BB-rat) with and without long-term administration of an aldose reductase inhibitor. Their findings of a significant, but incomplete prevention of neuropathy in these animals by aldose reductase inhibition suggest that additional mechanisms besides polyol-pathway activation may be of importance in the pathogenesis of diabetic neuropathy. Carrington and coworkers (1991) studied effects on impulse conduction after aldose reductase inhibition with imirestat in sciatic nerves of streptozotocin-diabetic rats both *in vivo* and *in vitro*. Cameron and coworkers (1991) studied nerve blood flow monitored by microelectrode polarography and hydrogen clearance in experimental diabetes in rats in relation to conduction deficits. Using these methods, Cameron and Cotter (2003) studied the effects of 5-hydroxytryptamine 5-HT₂ receptor antagonists on nerve conduction velocity and endoneurial perfusion in diabetic rats.

Schmidt and coworkers (1991) performed ultrastructural studies in mesenteric nerves of streptozotocin-induced diabetic rats.

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K.8.3

Nerve Blood Flow (Doppler Flux)

PURPOSE AND RATIONALE

In addition to nerve conduction studies, Calcutt and coworkers (1994) described a method to measure nerve blood flow by laser Doppler flowmetry in rats with streptozotocin induced diabetes treated with aldose reductase inhibitors.

PROCEDURE

Diabetes is induced in female Sprague-Dawley rats weighing 200–280 g by a single i.p. injection of 50 mg/kg streptozotocin after an overnight fast and is confirmed 2 days later by blood glucose determinations. Aldose-reductase inhibitor treated rats receive the test compound orally by gavage once a day for 2 months. For the experiment, the animals are anesthetized with an intraperitoneal injection (2 ml/kg) consisting of pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in 0.9% NaCl. For estimation of nerve blood flow, a laser Doppler flowmeter (TSI model BPM 403A, MN) with a wavelength of 780 nm is used. Flow determinations are made with a filter frequency of 30 Hz to 18 kHz and, depending on the magnitude of the reflected signal, one of four band widths (30 Hz to 1.3 kHz, 30 Hz to 3 kHz, 30 Hz to 7.5 kHz, or 30 Hz to 18 kHz). The flow meter continuously displays a moving average for the preceding 5 s in units of $\text{Hz} \times 10^2$. For flow measurements, the sciatic nerve is exposed and the probe tip of the flow-meter placed 1 mm above the mid-thigh region of the nerve using a micromanipulator. After a 10 s stabilization period, the first measurement is recorded. The probe is then advanced 1 mm distally for a second measurement and this process repeated until 10 readings are recorded over 1 cm of nerve. The mean of 10 values is taken as average nerve laser Doppler flow. Blood flow velocity, expressed in m/s, is reduced in diabetic rats.

EVALUATION

The effects of streptozotocin diabetes and of aldose reductase treatment are assessed by one factor analy-

sis of variance (ANOVA). Individual post-hoc comparisons are made with the Newman-Keuls method when the *F* ratio is $P < 0.05$.

K.8.4

Electroretinogram

PURPOSE AND RATIONALE

Diabetic neuropathy is one of the important symptoms of long-lasting diabetes (Engerman 1989). Several animal studies with aldose reductase inhibitors have been performed (Lightman et al. 1987, Hotta et al. 1988, Nagata and Robison 1988). Segawa and coworkers (1988) measured the development of electroretinogram abnormalities and the possible role of polyol pathway activity in diabetic hyperglycemia and galactosemia in the rat.

PROCEDURE

Male Sprague-Dawley rats weighing 310–400 g are dark-adapted for 20 min and anesthetized with i.p. injections of ketamine, 50–80 mg/kg and atropine sulfate, 2 mg/kg. Electroretinography is performed monocularly with the pupil maximally dilated. Photic stimulation is delivered with an intensity of one J in 20 s interstimulus intervals. Using a contact lens-type electrode, electroretinograms (ERG) evoked by strong flashes are amplified by a preamplifier, displayed on an oscilloscope and summed using a signal averager which also provides a copy of the averaged ERG. The amplitudes of the a- and b-waves are measured from the base line to the trough of the a-wave and from the trough of the a-wave to the crest of the b-wave, respectively. The amplitudes of the oscillatory potentials are measured. The peak latencies are measured as the intervals between the stimulus onset and the peak of the corresponding a- and b-waves and oscillatory potentials. The oscillatory potentials (designated O₁, O₂, and O₃ in order of appearance on the ascending limb of the b-wave) are added together. The sum of these amplitudes is expressed as the wavelet index.

For inducing galactosemia, male Sprague-Dawley rats weighing 140–185 g at the beginning of the study, receive a diet of 30% galactose. Test compounds are given as 0.1% to the diet.

EVALUATION

Data are calculated as mean \pm SEM and significance levels are estimated using the Wilcoxon rank sum test for unpaired data (two-sided). Linear regression is calculated by the least square method. A *p*-value of < 0.05 is regarded as statistically significant.

MODIFICATIONS OF THE METHOD

De la Cruz et al. (2003) studied the effects of clopidogrel and ticlopidine on experimental ischemic retinopathy in streptozotocin-diabetic rats. The percentage of the retinal surface occupied by horseradish peroxidase-permeable vessels was recorded.

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K.8.5**Streptozotocin-Induced Cataract****PURPOSE AND RATIONALE**

The increased incidence of cataracts, i. e. changes in the transparency or of the refractory index of the lens, in diabetic patients is well known. Evidence has accumulated for the involvement of polyol metabolism and the enzyme aldose reductase in diabetic cataractogenesis (van Heyningen 1959, Pirie and van Heyningen 1964). The enzyme aldose reductase catalyzes the reduction of aldoses such as glucose and galactose to the corresponding polyols, i. e., sorbitol and dulcitol. Sugar-induced cataractogenesis has been shown to parallel lenticular polyol accumulation (Kinoshita 1965). Since polyols do not readily diffuse through in-

tact cellular membranes, they create a severe osmotic stress within the lenticular cells which leads to cellular swelling and loss of integrity of the cellular membrane (Kinoshita 1974). Aldose reductase inhibitors have been shown to prevent sugar induced cataracts (Dvornik et al. 1973, Varma and Kinoshita 1976, Kinoshita et al. 1979, Muller et al. 1985).

PROCEDURE

Experimental diabetes is induced in Wistar rats weighing 150–180 g by intravenous injection of 50 mg/kg streptozotocin (Griffin et al. 1984).

EVALUATION

The values of animals treated with the aldose reductase inhibitor are compared with the values of rats treated with streptozotocin only.

MODIFICATION OF THE METHOD

Instead of systemic application, the aldose reductase inhibitor is applied twice daily for 6 weeks in solution as eye drops to female Sprague-Dawley rats with a starting weight of 120–140 g after diabetes induction with 70 mg/kg streptozotocin i.v. Body weight and blood sugar are registered twice a week. The progression of cataractous changes is followed by slit-lamp and Scheimpflug photography analysis every second week. At the end of the experiment, the animals are sacrificed and the lenses analyzed for their content of ATP, ADP, AMP, glucose, sorbitol, fructose, glucose-6-phosphate, and fructose-6-phosphate.

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K.8.6

Naphthalene-Induced Cataract

PURPOSE AND RATIONALE

Systemic application of naphthalene induces cataract in Brown-Norway rats. Aldose reductase inhibitors are tested for prevention of the cataract changes by parallel systemic or topical application (as eye drops).

PROCEDURE

Female Brown-Norway rats at an age of 6–7 weeks with a starting weight of 70–100 g are used. They are fed standard lab chow and receive water *ad libitum*. Naphthalene is dissolved in warm paraffin oil (10 g/100 ml) and is administered orally by gavage every second day in a dose of 1 g/kg. The test compound is administered orally every day or applied topically as suspension every day once, twice or four times to the right eye. The duration of the treatment is 6 weeks. Slit lamp microscopy (Zeiss photo slit lamp) during mydriasis with 1% atropine eye drops is performed in weekly intervals, including a baseline examination before the start of the treatment and a final examination prior to sacrifice. Scheimpflug photography of the anterior eye segment (Topcon SL-45 Scheimpflug camera) is carried out at the baseline examination, after 3 weeks of treatment and at the end of the experiment. Prior to sacrifice blood samples are taken by cardiac puncture under ether anesthesia. After sacrifice, lens fresh weight, concentration of oxidized and reduced glutathione, and the concentration of the aldose reductase inhibitor in the lenses and in blood samples are determined.

EVALUATION

The values of animals treated with the aldose reductase inhibitor are compared with the values of rats treated with naphthalene only.

MODIFICATIONS OF THE METHOD

Rathbun and coworkers (1996a) induced rapid-onset cataracts in SPF C57bl/6 mice by i.p. administration of naphthalene following cytochrome P-450 induction with phenobarbital. Rathbun and coworkers (1996b) assessed the activity of a L-cysteine prodrug suitable for glutathione biosynthesis rat lenses *in vitro* and as an agent for the prevention of acetaminophen-

and naphthalene-induced murine cataract in genetically susceptible mice. Holmen and coworkers (1999) compared different methods of photographic evaluation of cataract formation in rats in response to different regimes of naphthalene treatment.

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K.8.7

Determination of Advanced Glycation End Products (AGE)

GENERAL CONSIDERATIONS

Common to both type I and type II diabetes is the development of inflammatory and vascular complications that, over time, might portend significant morbidity and early mortality in affected subjects. Although multiple studies have suggested a direct role for adverse effects of glucose itself in modulating cellular properties, both in the extra- and intracellular milieu (Greene et al. 1987, Inoguchi et al. 1994, Xia et al. 1994, Nishikawa et al. 2000), recent observations suggest an emerging role for the products of non-enzymatic glycoxidation of proteins and/or lipids, the advanced glycation endproducts (AGE), in the pathogenesis of diabetic complications.

Formation of AGE

The non-enzymatic glycation and oxidation of proteins and/or lipids is a well-recognized phenomenon that occurs naturally, albeit at a very slow rate, during the course of aging. However, certain conditions within the cellular environment might enhance the formation of these modified structures. Specifically in the context of diabetes, hyperglycemia is a major trigger for the accelerated formation and deposition of

AGEs (Brownlee 1995, Ruderman et al. 1992). Multiple specific AGE, formed by glycation and/or oxidation, such as carboxy(methyl)lysine (CML), pentosidine and pyralline, have been identified in diabetic tissues (Ikeda et al. 1996, Reddy et al. 1995). It is speculated that, in diabetes, hyperglycemia alone might not be the sole perturbant driving formation of these modified structures, as AGE formation is enhanced in pro-oxidant states, such as that observed in diabetes itself (Baynes 1991). The observation that products of glycooxidation have been noted in hyperlipidemic atherosclerotic lesions, even in euglycemia, supports this concept. A recent suggestion is that *in vitro* AGE formation might be driven by the myeloperoxidase–hydrogen peroxide–chloride system, thereby providing a mechanism for conversion of hydroxy-amino-acids into glycolaldehyde, a precursor in the steps leading to formation of CML. Thus, these findings could have implications for AGEs in inflammatory settings, once confirmation *in vivo* has been established. It is important to note that, in diverse conditions, such as renal failure and Alzheimer's disease, AGE epitopes have also been identified within affected structures (Miyata et al. 1996), thereby providing a contributory mechanism for the cellular stress observed in these settings.

AGE and Mechanisms of Cellular Perturbation

It is likely that AGEs exert their effects *via* a number of mechanisms. Accumulation of cross-linking AGEs that tend to become insoluble, such as pentosidine, are thought to contribute to the tissue perturbation and rigidity that typify advanced diabetes, especially in the skin and basement membranes (Sell et al. 1992, Beiswenger et al. 1993). These factors are potentially linked to vascular leakiness and hyperpermeability. Vlassara and coworkers (1995) identified galectin-2 as a high-affinity binding protein for AGE. In addition, work from M. Brownlee's laboratory suggests that intracellular formation of AGE might modify the activity of growth factors, such as basic fibroblast growth factor.

Beyond the direct effects of AGEs on modulating cellular structure and function are those effects postulated to occur during ligation of specific cell surface receptors. In this context, the best-characterized receptor for AGEs (RAGE) is a multi-ligand member of the immunoglobulin (Ig) superfamily of cell surface molecules (Schmidt et al. 1992, Neeper et al. 1992). RAGE is composed of an extracellular region containing one 'V'-type Ig domain, followed by two 'C'-type Ig domains. This portion of the molecule confers the

ligand-binding properties, which are probably specific to the 'V'-domain. This portion of the molecule is followed by a hydrophobic transmembrane-spanning domain and, lastly, by a highly charged, short cytosolic domain that is essential for RAGE-mediated cellular effects upon engagement of ligand (Kislinger et al. 1999a, b; Yamamoto et al. 2001; Arancio et al. 2004; Hadding et al. 2004).

PURPOSE AND RATIONALE

Interactions between AGE and their binding proteins lead to the activation of a range of secondary messenger systems and increases the production of cytokines, including TGF- β , PDGF, and IL-1 (Ikeda et al. 1996). Recently, compounds that can cleave established AGE cross-links (Reddy et al. 1995), such as ALT711, a stable 4,5-dimethylthiazolium derivative of the prototype compound *N*-phenyl-thiazolium bromide, have been investigated. *In vivo* efficacy has been demonstrated in animal models, showing that these compounds can result in reduced AGE accumulation and can reverse age- and diabetes-dependent increases in arterial stiffness (Baynes 1991). RAGE as target for the prevention and treatment of the vascular and inflammatory complications of diabetes has been critically reviewed by Schmidt and Stern (2000a, b). The following assays enable the study of the AGE-RAGE interaction including its cellular effects as well as the modulation of both processes by compounds/drug candidates, which may be helpful for future therapy of diabetic late complications.

PROCEDURE

Cell Culture

The well-characterized normal rat kidney epithelial cell line (NRK-52E) can be obtained from the American Tissue Culture Collection. NRK-52E cells are believed to be of a proximal tubular origin on the basis of patterns of collagen secretion, C-type natriuretic peptide secretion, and the presence of EGF receptors. Cells are maintained in DMEM containing 4.5 g/l glucose with 10% FCS at 37°C in a 5% CO₂ atmosphere and passaged twice a week.

In Vitro Preparation of AGE Ligands

Procedures for the preparation of AGE ligands have been given by Oldfield and coworkers (2001) and Kislinger and coworkers (1999a and b). For preparation of AGE-BSA and AGE-RNase, BSA (10 mg/ml) or RNase (10 mg/ml) is incubated at 37°C for 6 weeks with D-glucose (90 g/l) in a 0.4-M phosphate buffer

containing azide (Vlassara et al. 1985). Control preparations are treated identically except that glucose is omitted. Preparations are extensively dialyzed against phosphate buffer to remove free glucose. The extent of advanced glycation is assessed by characteristic fluorescence (excitation 370, emission 440 nm). Advanced glycation is associated with an approximately 10-fold increase in fluorescence compared with controls.

For preparation of human AGE-IgG, human IgG (5 mg/ml in PBS) is subjected to non-enzymatic glycation by incubation in PBS containing 25 mM D-ribose. The solution is sterile-filtered (0.2 μ m) and then incubated at 37°C for 6 weeks under aerobic conditions. At the end of that time, the mixture is extensively dialyzed *versus* PBS at 4°C to remove unreacted ribose. This material is then chromatographed onto resin containing Affi-Gel 10 (Bio-Rad) to which had previously been adsorbed to recombinant human soluble RAGE. After incubation, the resin is washed extensively with 10-column volumes of TBS (20 mM Tris, pH 7.4, 100 mM NaCl). Bound components are eluted by 20 mM glycine (pH 2.5). The absorbance at 280 nm of each fraction is determined. Positive fractions are immediately neutralized and dialyzed *versus* TBS.

For preparation of carboxymethyl lysine–modified BSA (CML-BSA) (Kislinger et al. 1999a, b), 50 mg/ml aliquots of BSA are incubated with increasing concentrations of glyoxylic acid (5–90 mM) in the presence of approximately 5-fold molar excess of sodium cyanoborohydride. Control proteins are prepared under the same conditions, except that glyoxylic acid was omitted. The extent of chemical modification of lysine residues is determined as described previously using 2,4,6-trinitrobenzenesulfonic acid (Cayot and Tainturier 1997). The extent of lysine modification was up to 34% for CML-BSA preparations.

Iodination of AGE-BSA

AGE-BSA was iodinated by incubating AGE-BSA with chloramine-T (Greenwood et al. 1963). Bound 125 I was separated from free 125 I using a Biogel P6DG desalting gel (Bio-Rad Laboratories Inc.). Specific activity of the tracer is typically 400 Ci/mM.

Membrane Preparation

Membranes are prepared based on the method of Skolnik and coworkers (1991). NRK-52E cells are grown to confluence in 150-cm² tissue-culture flasks. Cells are washed twice with PBS, then detached from plates using a HEPES (100 mM) solution containing BSA (0.1%) and Triton X-100 (0.1%) with EDTA (5 mM), leupeptin (1 μ M), and PMSF (2 mM). Cells are cen-

trifuged (2,000 \times g, 5 min) and resuspended in the above buffer before disruption by ultrasound. Cell debris is removed by further centrifugation (2,000 \times g, 10 min) and the supernatant centrifuged (100,000 \times g, 1 h, 4°C). The supernatant is discarded, and the cell membranes in the precipitate are resolubilized in the above buffer. Protein concentrations are determined by the method of Bradford. The membrane preparation is used in binding studies and for ligand and Western blot analysis.

Binding Assay with Radiolabeled AGE

For binding studies according to the procedure of Oldfield and coworkers (2001), cell membrane extracts are incubated with 125 I-AGE-BSA (0.5 nM) and increasing concentrations of unlabeled AGE-BSA (0.015–7.46 μ mol) in the absence or presence of compounds/drug candidates for 3 h at 4°C in HEPES (100 mM) binding buffer with BSA (0.1%), Triton X-100 (0.1%), leupeptin (1 μ M), and PMSF (2 mM). A Brandel cell filter (Biomedical Research and Development Laboratories) is used to separate bound and free radioligand. A Tris-HCl (10 mM) polyethylene glycol (6.6%) buffer is used to wash components through the apparatus onto glass filter papers, which are counted in a γ -counter for 1 min. Binding experiments are performed in duplicate in five separate experiments, and the specificity of binding is assessed in further experiments using unlabeled, unmodified BSA (1 μ mol) as the competitor. Binding data were analyzed using a specific binding program (LIGAND).

Binding Assay with Radiolabeled RAGE

Human soluble recombinant RAGE (sRAGE) prepared as described above is radiolabeled with 125 I by incubation with IODO-BEADS (Pierce) to a specific activity of ~4,000–5,000 cpm/ng protein. In all cases, after radioiodination, precipitation of the radiolabeled material in TCA exceeded 90%. CML modifications of proteins or native, unmodified proteins are loaded onto the wells of plastic dishes (5 ng/well) in bicarbonate/carbonate buffer (pH 9.6) and incubated for 16 h at 4°C. Material in the wells is aspirated, and unoccupied sites are blocked by incubation with PBS containing 1% BSA for 2 h at 37°C. Wells are washed twice with PBS containing 0.005% octyl β -glucoside. Radioligand binding assay is performed in PBS containing 0.2% BSA with increasing concentrations of 125 I-human sRAGE alone or in the presence of a 50-fold molar excess of unlabeled human sRAGE or compounds/drug candidates for 2 h at 37°C. At the end of that time, wells are washed rapidly five times with

washing buffer. Elution of bound material is performed in a solution containing 1 mg/ml heparin. Solution is aspirated from the wells and counted in a γ -counter.

EVALUATION

Equilibrium binding data are analyzed according to the equation of Klotz and Hunston (1984): $B = nKA/1 + KA$, where B indicates specifically bound ligand (total binding, wells incubated with tracer alone, minus nonspecific binding, wells incubated with tracer in the presence of excess unlabeled material), n indicates sites/cell, K indicates the dissociation constant, and A indicates free ligand concentration) using non-linear least squares analysis. Specific binding of CML-BSA to radiolabeled RAGE is further determined by subtraction of nonspecific binding (counts obtained upon binding of radiolabeled sRAGE to immobilized BSA) from that obtained upon binding of radiolabeled sRAGE to immobilized CML-BSA. In case of binding to immobilized BSA, counts are negligible and less than 10% that observed in the presence of CML-BSA. Pretreatment with either antibodies, soluble RAGE, or the potential competitors and competitors/drug candidates is performed for 2 h prior to binding assay. Sometimes, it may be helpful to use materials eluted from the RAGE-Affi-Gel 10 columns (after passage of AGE-IgG) isolated RAGE domains for testing as unlabeled competitor in the binding assay. For this, human RAGE cDNA encoding the V, C1, or C2 domain is inserted into the *EcoRI* site of pGEX4T vector containing GST. Fusion proteins, V-GST, C1-GST, and C2-GST, are expressed in *E. coli*, purified on a glutathione-Sepharose column, and cleaved with thrombin (Amersham Pharmacia). RAGE domains are then purified to homogeneity using glutathione-Sepharose and characterized by SDS-PAGE and amino-terminal sequencing prior to testing in the radioligand binding assay.

Ligand and Immunoblotting Analysis

Cell membrane extracts (20 μ g of membrane protein per lane) are subjected to non-reducing SDS (12–15%) PAGE and electroblotted onto nitrocellulose membranes (Hybond, Amersham Pharmacia). For ligand blotting the membranes are blocked overnight at 4°C in 10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween buffer containing 2.5% BSA, before incubation with 125 I-AGE-BSA (110 ng/ml) for 2 h at room temperature. After washing with 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% Tween, the membrane is exposed to Kodak Biomax MS film for 1–4 h. Receptor-ligand binding specificity is studied in competitive experi-

ments where unlabeled AGE-BSA, AGE-RNase, BSA, and RNase (all 100 μ g/ml) are added as competing ligands.

In immunoblotting experiments, the nitrocellulose membrane is incubated at room temperature for 1 h with a polyclonal goat Ab against human RAGE 1:2,000 (Soulis et al. 1997, Brett et al. 1993). Membranes are washed before a 15-min incubation with a biotinylated secondary antibody and a streptavidin-horseradish peroxidase conjugate. Immunoreactivity is detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Primary antibodies are omitted in experiments as negative controls and RAGE antibody specificity is confirmed by pre-incubation of the membrane with recombinant RAGE before the addition of RAGE antibody.

Isolation of Human Serum Albumin and Immunoblotting for AGE

Plasma samples are obtained from human donors with diabetes, renal failure, or age-matched healthy controls in accordance with the laws of the respective national ethics committees. Samples (2 ml) are dialyzed for 16 h at 4°C in 20 mM phosphate buffer (pH 7.1) in a total volume of 4 ml. After dialysis, samples are subjected to filtration (0.8 μ m), and then chromatographed onto columns containing Affi-Gel blue resin (Bio-Rad) previously equilibrated in 20 mM phosphate buffer (pH 7.1). 5 ml of resin are employed per ml of plasma. The resin is washed in 2.5 column volumes of phosphate buffer as above, and human serum albumin is eluted by application of phosphate buffer containing 1.4 M NaCl. The absorbance at 280 nm for each fraction is determined, and positive fractions are determined by SDS-PAGE followed by staining with silver. For immunoblotting, the materials (30 μ g of protein, each) are employed to SDS-PAGE gels (8%). Simultaneously, marker proteins are added. After electrophoretic separation and transfer of the proteins to nitrocellulose membranes, the unoccupied sites on the membranes are blocked in the presence of non-fat dry milk (13.5%) in TBS for 4 h at room temperature. Immunoblotting is performed using affinity-purified anti-CML IgG as above (10–20 μ g/ml) in milk buffer (5%) for 1.5 h at 37°C. Membranes are washed extensively in TBS containing 0.1% Tween 20. The membranes are incubated with goat anti-rabbit IgG labeled with horseradish peroxidase for 1 h at 37°C, washed extensively in the above buffer. Visualization of antibody binding was performed employing the ECL detection system (Amersham Pharmacia). Quantitative evalua-

tion of band intensity is performed using Molecular Dynamics/ImageQuant.

K.8.8

Measurement of Reactive Oxygen Species (ROS) Production

GENERAL CONSIDERATIONS

All forms of diabetes, both inherited and acquired, are typified by hyperglycemia, a relative or absolute lack of insulin, and the development of diabetes-specific microvascular pathology in the retina (retinopathy), renal glomerulus (nephropathy) and peripheral nerve (neuropathy). At the molecular cell biology level, three major hypotheses regarding the mechanisms by which hyperglycemia causes diabetic complications, i. e. the aldose reductase pathway (see above), formation of AGE (see above) and activation of PKC, have each generated a great deal of supporting data (Brownlee 1995, Koya and King 1998, Schmidt and Stern 2000b). Seminal work from Michael Brownlee's laboratory has shed new light on the mechanisms by which elevated concentrations of glucose perturb cellular properties in a fundamental way, thereby joining these three pathways into a unifying hypothesis for hyperglycemia-induced late complications (Nishikawa et al. 2000). In endothelial cells subjected to physiologically relevant glucose concentrations as a model system, the non-insulin-dependent uptake of glucose *via* GLUT1 leads to oxidative stress (OS) with concomitant generation of reactive oxygen species (ROS), the increase of both is positively correlated to the glucose concentration. Several pathways are considered as likely candidates for the emergence of OS and the formation of oxygen free radicals in cells, including the accelerated flux of glucose through glycolysis and feeding of pyruvate, thus formed, to the tricarboxylic acid cycle, which overloads mitochondria and thereby causes excessive generation of ROS. In turn, OS and ROS has recently been shown to cause upregulation of the mitochondrial uncoupling protein 2 (UCP-2), leading to a vicious cycle of increased electron flux *via* the respiratory chain and concomitant elevated production of ROS. Most importantly, it has been demonstrated recently, that suppression of intracellular ROS, using low molecular weight inhibitors or by expression of the anti-oxidant enzyme, manganese-dependent superoxide dismutase (MnSOD), prevents each of the three molecular mechanisms mentioned above (Nishikawa et al. 2000). Consequently, glucose-induced formation of oxidants is a proximal step in cellular perturbation prevalent during diabetes. Thus, assays which moni-

tor the generation of OS and ROS in response to compounds/drug candidates may be helpful for the finding and characterization of future anti-diabetic drugs, which may reduce the risk for the development of diabetic late complications.

PURPOSE AND RATIONALE

Although there are various methods to assess oxidative damage of cells, such as measuring lipid peroxidation products and DNA adducts (Holley and Cheeseman 1993), none of them evaluate the OS directly. With the first description of using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a fluorometric assay for hydrogen peroxide (Keston and Brandt 1965), it became popular to use dichlorofluorescein (DCFH) as a probe to evaluate intracellular hydrogen peroxide formation by flow cytometry. The theory behind using DCFH-DA is that nonfluorescent fluorescein derivatives will emit fluorescence after being oxidized by hydrogen peroxide (LeBel et al. 1992). The emitted fluorescence is directly proportional to the concentration of hydrogen peroxide. When applied to intact cells, the nonionic, nonpolar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH (LeBel et al. 1992, Bass et al. 1982). In the presence of reactive oxygen species (ROS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) (LeBel et al. 1992). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall OS in cells.

Many studies have used fluorescent microscopy to quantify OS in cells using DCFH-DA. This method has the problem of inducing photooxidation of DCFH to DCF intracellularly and emitting fluorescence, because it is difficult to control the time of light exposure when trying to locate and focus cells under the microscope. In addition to the problem of photooxidation, there is no standard way to quantify the OS using a microscope. In order to prevent the overestimation of the OS due to photooxidation, an instrument equipped with fast light excitation and fast fluorescence capturing, which will not induce excessive photooxidation, is needed. Wang and Joseph (1999) have introduced a fluorescent microplate reader-based method to evaluate OS in cultured cells, induced by applying various free radical generators extracellularly, using DCFH as the probe.

PROCEDURE

Measurement of OS

Cells (e. g. PC12) are grown in growth medium containing 85% RPMI-1640 with *l*-glutamine, 10% heat-

inactivated horse serum, 5% FBS, 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. The cells are maintained in collagen-coated plates in 5% CO₂/95% air at 37°C. The culture medium is changed twice every week and the cells are split 1:4 or 1:8 every week. Cells were split and counted by trypan blue exclusion. Viable cells (10⁴/well) are plated into 96-well collagen-coated plates one day before the experiments. On the day of the experiments, after removing the medium, the cells in the plates are washed with KRH buffer and then incubated with 100 µM 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes Inc. USA) in the loading medium in 5% CO₂/95% air at 37°C for 30 min. For loading the cells with DCFH, DCFH-DA from a 10-mM stock solution in DMSO is mixed with loading medium (99% RPMI-1640 and 1% FBS) to a final concentration of 100 µM. After DCFH-DA is removed, the cells are washed and incubated with KRH buffer (with different concentrations of one of the free radical generators, e. g. H₂O₂, dopamine) in the absence or presence of high glucose concentration. The fluorescence of the cells from each well is measured and recorded.

For OS measurement, DCFH-DA-loaded cells are placed in a CytoFluor Series 4000 multiwell fluorescence plate reader with temperature maintained at 37°C. The excitation filter is set at 485 nm and the emission filter is set at 530 nm. The fluorescence from each well is captured, digitized, and stored on a computer using Cytofluor (Version 4.0) (PerSeptive Biosystems Inc. USA). Data points are taken every 5 min for 30 min and the data are exported to Excel (Microsoft, USA) spreadsheet software for analysis.

Measurement of ROS

For measurement of ROS production according to the procedure of Freeman and coworkers (2006), a 10-mM stock solution of H₂DCFDA-SE (2',7'-dichlorofluorescein diacetate with a succinimidyl ester group, DCF, Molecular Probes) is prepared in DMSO, stored at -70°C, and diluted with KRB (pH 7.0) just before use to a final concentration of 10 µM. The cells are plated in 24-well cell culture plates overnight. They are then washed twice with PBS, incubated in PBS plus DCF for 30 min at 37°C, washed with PBS, and incubated in KRB containing 5.5 or 20 mM glucose for a further 30 min. They are imaged at room temperature using an IonOptix fluorescence system, with 495 nm excitation and 520 nm emission. The background fluorescence is subtracted. 0.5 mM menadione is used as a generator of ROS as a control. The Ros concentrations are determined from a standard curve

of H₂O₂ (5–50 µM). To confirm that glucose generates ROS, dihydroethidium (DHE) conversion to ethidium by oxidation can be measured according to the procedure of Russell and coworkers (2002). The ratio of DHE:ethidium is determined using a fluorimeter and standardized against cellular protein. DHE is measured using 355 nm excitation and 430 nm emission, and ethidium is measured using 518 nm excitation and 605 nm emission.

Measurement of Mitochondrial ROS Production

To evaluate the direct production of mitochondrial ROS in cells, the DCFDA measurements have to be combined with ROS-specific staining by using the reduced Mito Tracker Red probe (CM-H₂Xros; Molecular Probes Inc. USA) according to the procedure described by Degli Esposti and coworkers (1999). For this, the cells suspended in growth medium at 1 × 10⁶/ml are incubated for 15 min at room temperature with freshly prepared CM-H₂XRos (0.5 µM), then washed twice with PBS, and collected on a slide using a Cytospin apparatus (Wolvetang et al. 1994). The cells are fixed with 3.7% formaldehyde in PBS, followed by washing first with PBS containing 30 mM NH₄Cl and then with distilled water. In some experiments, the fixed cells are counterstained with Hoechst 33342 before mounting with anti-fade medium, and the slides are stored at 4°C in the dark. In parallel experiments, mitochondrial staining to analyze membrane potential can be performed using 100 nM oxidized Mito Tracker Red (CM-XRos) following the same protocol as outlined above for CM-H₂XRos. Confocal microscopy is undertaken with a Krypton/Argon instrument, usually with a 40× oil objective and intermediate laser and photomultiplier voltage. The 590-nm bandpass filter is used to detect the red fluorescence of CM-XRos staining (Wolter et al. 1997). Epifluorescence is evaluated with a microscope using a Texas Red filter and a CCD camera attached to a microcomputer image analyzer.

EVALUATION

The percentage increase in fluorescence per well is calculated by the formula $[(F_{t30}-F_{t0})/F_{t0} \times 100]$, where F_{t30} = fluorescence at time 30 min and F_{t0} = fluorescence at time 0 min. This method of analysis has advantages over analyzing just the net change in fluorescence in that, not only did the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well, they also effectively control for variability among wells. This method also cancels out the background

fluorescence in each well, and therefore, a “no cell” control is not needed.

Various free radical generators produce concentration-dependent changes in DCF fluorescence, indicating the indiscriminate nature of DCF. Due to the indiscriminate nature of DCFH, which can be oxidized by various ROS and not just H₂O₂, the increase of intracellular DCF fluorescence does not necessarily reflect the levels of ROS directly, but rather an overall OS index in cells. Quantifying cellular OS by the DCF assay using a fluorescent microplate reader is an easy and efficient method with low variability which can be used to quantify the potency of pro-oxidants or can be adapted to evaluate the efficacy of antioxidants against ROS in various cell lines. The use of a 96-well microplate reader enables the generation of a large amount of data with low variability.

Measurement of Mitochondrial Size

Since glucose-induced ROS production and swelling of mitochondria seem to be correlated (Russell et al. 2002), determination of alterations in the size of mitochondria exposed to OS may be indicative for the generation of mitochondrial ROS. For measurement of the mitochondrial size, cells are loaded with Mitotracker orange (CMTMRos; Molecular Probes Inc. USA) using confocal microscopy with 540 nm excitation. Each mitochondrion is a complex fractal-like network, making accurate analysis of volume difficult. Therefore, the maximum cross-sectional area is determined from identification of each individual mitochondrion throughout the z-series. All distinguishable mitochondria at each step level in the z-series are measured at the largest cross-sectional area of the mitochondrion. The size of the mitochondria is determined using the perimeter of visible CMTMRos and reflects the mitochondrial area at the measured level. Identical methods of identification and measurement are applied to each mitochondrion in each cell and in each condition, thus reducing the effect of technical artifact. The mitochondrial areas are then averaged for each neuron and, where necessary, results are first converted to a log normal distribution before obtaining descriptive statistics or performing tests of inference about the data set. Results are obtained for > 100 mitochondria in at least 10 randomly measured neurons per condition.

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K.9

Insulin Analogs: Assessment of Insulin Mitogenicity and IGF-I Activity²

K.9.1

Introduction and Application to Insulin Analogs

The metabolic activity of insulin has been studied extensively in-vitro and in-vivo, based on the initial assessment of insulin receptor affinity, followed by methods to estimate the metabolic activity in vitro. These estimates provides some guidance about the biological activity which will be found in-vivo, they need to be confirmed and supplemented by testing the glucose lowering activity in animals (mice, rats, dogs, pigs). The biological effects (hypoglycemic activity) are related to the direct activation of the insulin receptor and subsequent signaling through intracellular mechanisms. The second group of biological effects is related to cell proliferation (mitogenic activity), which may be mediated by the insulin receptor, by the IGF-I receptor and by hybrids of the two receptors. The evaluation of the relevance of mitogenicity estimates may be performed in in vitro and in-vivo. One approach is cell proliferation in benign and malignant cell lines, for example on mammary epithelial cell lines MCF-10 and MCF-7 (Milazzo et al. 1997).

In the in-vivo evaluation, the metabolic activity is determined in animals (see chapter K.3) using the rat or rabbit bioassay (Lin et al. 1999) by intravenous injection, to confirm the blood glucose lowering activity after direct receptor activation. This initial test confirms intrinsic activity at the receptor level, However, it does not indicate depot activity and is applicable in the same manner to fast acting insulin analogs (lispro, aspart, glulisine), and to long-acting analogs (glargine, detemir). The rat bioassay by intravenous injection is preferred is the initial test for in-vivo biological activity. In order to evaluate depot activity, the time action profile after subcutaneous injection may be determined subsequently, in order to distinguish between the rapid acting insulin analogs and long acting insulin analogs. This has been done in clinical studies by measuring the glucose lowering, and the residence of ¹²⁵I-labeled insulin analogs at the subcutaneous injection site (Kang et al. 1991a).

²Amendment by J. Sandow

In a practical approach, testing for depot activity may be performed by the pharmacodynamics of glucose lowering, preferably in intact dogs. Such studies may be later followed up by euglycemic clamp studies. The pharmacokinetics can be based on non-specific measuring of immunoreactive insulin, and concentration changes of C-peptides. More specific determination of the insulin analog concentrations require advanced methods, for example there is the specific radioimmunoassay for lispro insulin (Bowsher et al. 1999, von Mach et al. 2002).

GENERAL CONSIDERATIONS

A general approach may be suggested for the *in vitro* evaluation of new insulins. This approach is based on the consideration that insulin has metabolic as well as mitogenic activity which is mediated predominantly by signaling via the insulin receptor, and at high concentrations - as used *in vitro* - may also be mediated by signaling via the IGF-I receptor. The primary step is determination of insulin receptor affinity, followed by IGF-I receptor affinity (Baehr et al. 1997). There is one important reference compounds, [B10-Asp] insulin which should be used in these evaluations as comparator (Berti et al. 1998), because of the toxicological findings with [B10-Asp] insulin which have raised concern about enhanced mitogenic activity of insulins with prolonged residence time at the insulin receptor (Jorgensen et al. 1992). [B10-Asp] insulin was evaluated as fast acting insulin analog in early clinical trials (Kang et al. 1991b, Nielsen et al. 1995), and was subsequently found to induce benign and malignant mammary gland tumours in rats, during twelve-month toxicology studies. This has been attributed to enhanced mitogenic signaling via the insulin receptor, and alternatively to increased affinity for the IGF-I receptor with subsequent growth promoting effects on mammary gland tissue. A guideline regarding the evaluation of new insulin analogs for carcinogenicity was issued by the European Agency for the Evaluation of Medicinal Products (EMA 2001), with specific guidance about the *in vitro* and *in vivo* evaluation to be performed. In this guideline, the specific reference compounds mentioned a human insulin, [B10-Asp] insulin and IGF-I (somatomedin C).

PURPOSE AND RATIONALE

The structure and approach to the evaluation of new insulins is directed towards efficacy in terms of glucose lowering, in the predictive manner to differentiate between monomeric insulins with fast absorption kinetics,

and long acting insulins with delayed absorption from the subcutaneous injection site.

PROCEDURE

The initial steps are performed *in vitro*, focused on receptor interaction. Subsequent steps *in vitro* to explore the receptor-mediated signaling, which is however at the present time difficult to attribute precisely to biological and clinical effects. This is due to the wide concentration range permissible during *in vitro* studies, whereas the narrowly limited clinical concentration range needs to be kept in mind during any interpretation of clinical relevance (Zib and Raskin 2006).

The main aim of the *in vitro* evaluation is to establish the relation of metabolic activity to mitogenic activity, as previously done for structure activity studies on insulin analogs, and subsequently directed to predicting clinical relevance of these observations, when comparing new compounds with the established biochemical and toxicological profile of [B10-Asp] insulin, and the clinically used fast acting and long-acting insulin analogs.

MODIFICATIONS OF THE METHOD

The primary step of measuring insulin receptor affinity may be performed in several modifications, depending on the receptor preparation, and on the source of insulin receptors e.g. from fibroblasts, placental membranes, skeletal muscle cells and other sources. The second step of measuring IGF-I receptor affinity also has several options, for research on the IGF-I receptor a human osteosarcoma cell line (SAOS B10) was in several instances applied due to the high number of IGF receptors and lower number of insulin receptors on the cells (Kurtzhals et al. 2000).

The biological profile in animals is directed at predicting clinical utility either as a fast acting (meal-time) insulin, or at establishing depot activity in a suitable animal species. Blood glucose lowering (hypoglycemic) activity is also important for the design of safety studies depending on the preclinical metabolic to mitogenic ratio. In the chronic toxicity studies, and where applicable in carcinogenicity studies, the dose range from biologically effective dose to maximum tolerated dose may be 50–100fold, the results than need to be interpreted carefully for their relevance in relation to the therapeutic concentrations found in clinical therapy.

CRITICAL ASSESSMENT OF THE METHOD

When considering the extensive knowledge on three fast acting insulins and two long acting insulins cur-

rently in clinical use, the predictive relevance of the methods described here can be assessed in each case, and selection of the appropriate study design is based on existing clinical experience.

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K.9.2

Insulin Receptor Affinity

GENERAL CONSIDERATIONS

An initial step in the evaluation of insulin analogs is determination of their affinity for the insulin receptor (Lee and Pilch 1994). The studies may include receptor association and dissociation as well as the signalling via the insulin receptor (insulin receptor tyrosine kinase activity and tyrosine phosphorylation of substrates). These methods provide some guidance about the biological potency to be expected, although they provide only limited information about the time action profile.

Receptor affinity data to be extended by evaluation of post-receptor events (signaling), The time action profile in animals and humans, which may differ considerably from the predictions derived from the receptor affinity alone.

PURPOSE AND RATIONALE

These methods described the initial binding of insulin analogs to the receptor preparation obtained from different sources. Tissues differ in their receptor content, the early methods used membrane receptors isolated from different organs, and more recent methods use solubilised human insulin receptor protein.

PROCEDURE

There are several important modifications of the assay principle. This depends on selection of the initial source of receptor (tissue), the preparation of the receptor protein, and the procedure to determine receptor affinity using a radioactive labeled insulin preparation. Some details of the methods are described in section K.6.3.2.1.1 of this chapter.

Sliker et al. 1997 described the method using placental membranes and mammary epithelial cells as the receptor preparations for [¹²⁵I]-Insulin and [¹²⁵I]-IGF-I binding assays. The placental assay employed incubating 30–40 mg of membrane protein with approximately 10 fmol of iodinated ligand in a final volume of 500 µl of 100 mmol/l HEPES, pH 7.8, 120 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l MgSO₄, 8 mmol/l glucose and 0.25% BSA for 16–18 h at 4°C. Membranes were collected on glass fiber filters pre-treated with 0.1% polyethyleneimine by using a cell harvester (Skatron, Lier, Norway). Binding assays with mammary epithelial cells were performed under similar conditions using confluent cells on either P12 (IGF-I receptor) or P6 (insulin receptor) plates (Costar, Cambridge, Mass., USA). Monolayers were incubated in insulin-free medium for 24 h prior to initiating the binding assay. Cells were incubated in the above buffer containing iodinated IGF-I and cold competing ligands for 16–18 h at 4°C. After washing the monolayers with cold assay buffer, cells were solubilized in 0.1 N NaOH and counted for ¹²⁵I. EC₅₀ values were determined by fitting displacement data to a four-parameter model by non-linear regression. Dissociation constants for TyrA14-[¹²⁵I]-Insulin, -LysB28ProB29 HI, and -AspB10 HI were obtained according to the method of Drejer et al. (1991).

For the quantification of receptor numbers of insulin and IGF-I on Chinese hamster ovary cells (CHO) using the [¹²⁵I] labeled ligands, Hansen et al. (1996) applied the method which was also modified for time course studies of binding to these receptors.

CHO-hIR and CHO-K1 cells were seeded on 24-well Nunclon plates at a density of 5 × 10⁵ cells/well in DMEM containing 20 mM Hepes, 1 µg/l human albumin, 10% fetal calf serum, 1% non-essential amino acids and 0.1% bacitracin (pH 7.4); cells were used at confluence. The numbers of insulin and IGF-I receptors present at the surface of the CHO-K1 and CHO-hIR cells were determined by saturation of the respective receptors with unlabeled ligand. Cells were incubated overnight at 4°C with 50 pM ¹²⁵I-labeled insulin or [¹²⁵I]IGF-1 in the presence of increasing amounts of unlabeled insulin or IGF-1 respectively. The number of receptors was estimated with the aid of the LIGAND program fitting a two-site model [Munson and Rodbard 1980]. The native CHO-K1 cells were estimated to express approx. 3,000 high-affinity binding sites for insulin and 50,000 IGF-1 receptors, whereas CHO-hIR cells were found to express 60,000 IRs and 50,000 IGF-1 receptors.

For the time course of receptor association, the procedure was as follows (Hansen et al. 1996). CHO-hIR cells were cultivated in DMEM containing 2 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 10% fetal calf serum, 1% non-essential amino acids and 1 µM methotrexate. Cells were grown to 80% confluence in 60 mm dishes, 6-well plates (IRTK activity assay) or 12-well plates before the experiments were carried out. Before stimulation with insulin or analog, the cells were starved for 18 h in serum-free medium supplemented with 0.5% insulin-free BSA. The cells were stimulated with 1 ml of 0.1 µM insulin or analog for 30 min, then washed thoroughly 3–5 times (2–5 ml each wash) and subsequently incubated in serum- and insulin-free medium. Because of the large variations in receptor kinetics and potencies of the analogs, the high concentration of 0.1 µM and the 30 min stimulation time were used to ensure maximal stimulation. At various times post-stimulation (0–180 min) the medium was removed and the cells processed further (insulin receptor tyrosine kinase activity IRTK and tyrosine phosphorylation of IR, IRS-1 and Shc).

Kurtzhals et al. (2000) used a preparation of human solubilised insulin receptor protein. Human insulin receptor (hIR) (isoform without exon 11) was isolated from transfected baby hamster kidney (BHK) cells by solubilization and partial purification on a wheat germ agglutinin column. For binding experiments, human insulin receptor (hIR) was incubated with 3 pmol/l TyrA14[¹²⁵I] human insulin and various concentrations of unlabeled human insulin or insulin analog in a binding buffer containing 0.1 mol/l HEPES, 0.1 mol/l NaCl, 0.01 mol/l MgSO₄, 0.5% HSA, 0.2% g-globulin, and 0.025% Triton X-100, pH 7.8, for 42 h at 4°C. Bound tracer was isolated by precipitation with 400 µl 25% PEG 8000 and washing with 1 ml 15% PEG 8000. The data were fitted to a 4-parameter logistic function where *B*_{max} and *B*_{min} were fixed and slope and concentration required for half-maximal effect (EC₅₀) varied. The relative affinity of an insulin analog was calculated as the ratio between the EC₅₀ value for human insulin and that of the insulin analog.

Dissociation from the insulin receptor was determined by Kurtzhals et al. (2000) using CHO cells. Dissociation constants for A14Tyr[¹²⁵I]-insulins were determined as previously described using Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor (CHOhIR cells) (9). The cells were cultured at 37°C in a 5% CO₂ humidified atmosphere in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 1% nonessential amino acids, 50 µg/ml streptomycin, 50 U/ml penicillin, and 1 µmol/l methotrexate. Cells were subcultured at a 1:5 split ratio every 3–4 days for maintenance. For dissociation studies, cells were seeded on 24-well plates at a density of 5 × 10⁴ cells/well in DMEM containing 10% FCS, 1% nonessential amino acids, 20 mmol/l HEPES, 0.1% HSA, and 0.1% bacitracin (pH 7.4). Cells were used at confluence and were incubated with 50 pmol/l A14Tyr[125I]-insulin or analog for 3 h at 4°C. Cells were washed quickly twice with ice-cold HEPES-buffered DMEM (pH 7.4), and the dissociation of radioactivity was measured after the addition of HEPES-buffered DMEM containing 0.1 µmol/l unlabeled human insulin to measure the maximal accelerated dissociation rate. Cell-associated radioactivity was measured as a function of time, and the dissociation rate constant (*k_d*) was calculated from the fitted monoexponential dissociation profiles using the following equation: $B = B_{\min} + (B_{\max} - B_{\min}) 3 \exp(-k_d t)$, where *B* represents cell-associated radioactivity.

MODIFICATIONS OF THE METHOD

The main modifications are related to the selection of receptor protein (membranes or solubilized receptor protein, cells transfected with the human insulin receptor – CHOIR cells)

CRITICAL ASSESSMENT OF THE METHOD

It is important to use the same system when comparing the insulin receptor affinity of several insulins. To some extent, this can be used as the selection tool in structure activity studies. There are however considerable limitations when comparing affinities with *in vitro* potency, and ultimately with *in vivo* hypoglycemic activity. Therefore, results of the insulin receptor affinity need to be supplemented by data on receptor association and dissociation, as well as post-receptor signaling mechanisms.

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K.9.3

Signaling Via Insulin Receptor

GENERAL CONSIDERATIONS

The postreceptor events after stimulation of the insulin receptor are of considerable interest for understanding the effects on different tissues, changes associated with insulin resistance and mitogenic activation of normal tissue and tumor tissues. This is a very complex area of research, because the mitogenic signalling is concentration dependent and many effects may be obtained *in vitro* which have no equivalent within the therapeutic range of insulin application in patients. Due to the complexities of the signaling mechanisms, and their redundancy with regard to cell proliferation, apoptosis and tissue growth, these investigations are necessary for better understanding, but not sufficient at the present time to fully explained the antiapoptotic and mitogenic activities *in vivo* and the clinical relevance.

PURPOSE AND RATIONALE

Studies on signaling of insulin analogs via the insulin receptor over a wide range of concentrations *in vitro* are performed to elucidate the intracellular mechanisms, and the components of the signaling chain.

PROCEDURE

Among the many procedures and protocols used for investigation of signaling in cells stimulated with insulin analogs, Hennige et al. (2005) provide an example for the assessment of the fast acting insulin glulisine. Their tests were performed in 10-week-old male C57BL/6 mice from Charles River acclimatized for 2 weeks before entering the study. The mice were maintained on a normal light/dark cycle and kept on a regular diet. Glucose levels were sampled from mouse tail bleeds using a Glucometer Elite (Bayer, Elkhart, IN).

The procedures for *in vivo* insulin stimulation and Western blot analysis were described for a short term experiment, by single dose stimulation with regular human insulin or insulin glulisine (1 IU/kg body wt) injected intraperitoneally. For short-term stimulation, 2 IU of insulin were injected into the inferior vena cava. Control animals received a comparable amount of diluent. Tissues (liver, muscle, and hypothalamus) were removed at the indicated time points and homogenized at 4°C, as previously described (8). Homogenates were allowed to solubilize for 30 min on ice and were clarified by centrifugation at 12,000g for 20 min. To detect insulin-stimulated tyrosine phosphorylation, supernatants containing 0.5 mg of total protein were immunoprecipitated with antibodies directed against the COOH-terminal sequences of the insulin receptor, and of the insulin receptor substrates IRS-1 and IRS-2. Visualization of immunocomplexes after gel electrophoresis and Western blotting with the antiphosphotyrosine antibody 4G10 was performed with the nonradioactive enhanced chemiluminescence system. Blots were subsequently stripped and reprobed to reveal expression of total protein. To assess activation of downstream signaling elements, tissue lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with anti-phospho-AKT and anti-phosphomitogen-activated protein (MAP) kinase antibodies (Cell Signaling, Beverly, MA), respectively.

Assay of PI 3-kinase activity: Tissue lysates were immunopurified with anti-PY20 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies and immunocomplexes were absorbed to protein a-sepharose for 12 h. Immunoprecipitates were washed three times and pellets were directly incubated with 0.1 mg/ml L-PI (Sigma) and 50 mol/l [32P]-ATP (Perkin Elmer) at room temperature for 10 min. After 150 l of 1 mol/l HCl was added, lipids were extracted twice with 450 l chloroform/methanol (1:1 by vol). Products were separated by thin-layer chromatography, as previously de-

scribed (9). ³²P-labeled phospholipids were detected by autoradiography.

[3H]thymidine incorporation into C2C12 myoblasts: To measure [3H]thymidine incorporation, C2C12 cells were grown in Dulbecco's modified Eagle's medium (4.5 g/l glucose and 10% FCS) in six-well culture plates, and subsequently starved for 24 h in serum-free medium. After cells were stimulated with insulin for 16 h, [3H]thymidine (0.5 Ci/ml) was added for 4 h. The dishes were then rinsed twice with ice-cold PBS and once with 10% trichloroacetic acid. After 20 min, dishes were washed once with ice-cold 10% trichloroacetic acid. Cells were then lysed with 500 l of 0.2 N NaOH/1% SDS and neutralized with 0.5 ml of 0.2 N HCl. Radioactivity was determined by liquid scintillation counting.

MODIFICATIONS OF THE METHOD

There are many options to analyze the postreceptor events to characterize signaling in insulin stimulated cells (Gronborg et al. 1993, Chang et al. 2004), with considerable overlap towards the IGF-I stimulated signaling (Valentinis and Baserga 2001). For the practical approach of risk assessment for insulin analogs, these methods are not suitable, due to the complexity and the as yet only partially defined relation to proliferative activity in normal tissues, and in pre-existing tumors. The research potential of these methods in defined cell lines in culture is very important, the application to insulin stimulated animals and their tissues is also of considerable heuristic relevance.

CRITICAL ASSESSMENT OF THE METHOD

Exploration of the insulin receptor related signaling mechanisms is at present an active research area with many a well-defined methods (see Sect. K.6 in this chapter).

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K.9.4

IGF-I Receptor Affinity

GENERAL CONSIDERATIONS

IGF-I receptor affinity is characterised by binding of IGF-I to receptors on cell membranes, or to the IGF-I receptor protein. The cell lines which have been selected for their high density of IGF-I receptors provide estimates of affinity which may vary considerably, and need to be supplemented by methods for interaction of insulin analogs with the receptor (dissociation, residence time), and receptor-mediated effects on proliferation in normal tissues, and in tumor cell lines.

For the IGF-1 receptor affinity of insulin analogs, cell lines have been used with a favorable relation of IGF-I receptors to insulin receptors. Clearly, results differ with the test system used, therefore the determination of IGF-I receptor affinity is considered a preselection step for the further investigation of signalling via the IGF-I receptor and the insulin receptor. Placental membranes or mammary epithelial cell lines (Sliker et al. 1997), and human skeletal muscle cells (Ciaraldi et al. 2001) have been used for structure activity studies, and full investigations on signaling (Ciaraldi et al. 2005),

PURPOSE AND RATIONALE

Studies are performed to obtain an estimate of the proliferative and antiapoptotic activity to be expected raised on the affinity for the IGF-I receptor (DeMeyts et al. 1994, Rubin and Baserga 1995), and to explore the mitogenic/metabolic ratio in preparation of detailed investigation of the effects on cells (e. g. thymidine incorporation), and on postreceptor signalling (Leroith et al. 1994).

PROCEDURE

Human skeletal muscle cells (Ciaraldi et al. 2001) are used for the assessment of insulin analog interaction with insulin receptors and IGF-I receptors.

Cell Culture

Biopsy of the vastus lateralis muscle was performed according to published procedures (19). Human skeletal muscle cells were isolated and grown in culture as described in detail previously (Henry et al. 1995). When myoblasts attained 80–90% confluence, the growth medium was changed to MEM supplemented with 2% FBS and antibiotics to induce fusion to multinucleated myotubes. The medium was changed every other day during cell fusion. All studies were performed on cells after one passage.

Insulin and IGF-I Binding Assays

Hormone binding assays were performed by the modification of a method described previously (Henry et al. 1995). Fully differentiated human skeletal muscle cells from nondiabetic subjects and patients with type 2 diabetes were washed four times with reaction buffer, then incubated with reaction buffer and [125I-Tyr A14]insulin (final concentration, 67 pm) or [125I-Tyr A14]IGF-I (final concentration, 39 pm) for 4 h at 12 C in the absence or presence of varying concentrations of unlabeled hormone. Results were calculated based on the displacement of specific insulin/IGF-I binding normalized to protein concentrations in cells from both nondiabetic subjects and patients with type 2 diabetes.

MODIFICATIONS OF THE METHOD

One particular assay based on IGF receptor affinity in an osteosarcoma cell line SAOS B10 was used extensively in the preclinical evaluation of mitogenic activity (Kurtzhals et al. 2000). This cell line was initially selected for research on IGF-I, and subsequently adapted to structure activity studies. The finding of slightly increased IGF-1 receptor affinity in the presence of a rate of receptor dissociation similar to human insulin is not associated with any biological consequences, as shown by the subsequent extensive studies on absence of toxicity and carcinogenicity mice and rats (Stammberger et al. 2002).

CRITICAL ASSESSMENT OF THE METHOD

It remains the highly active and controversial area of research to define which mechanisms are involved and contribute to a significant extent to cell proliferation and apoptosis via the insulin receptor and/or the IGF-I receptor (Blakesley et al. 1996, DeMeyts 1994, DeMeyts et al. 1995a, DeMeyts et al. 1995b). Evidence for these mechanisms relies to significant extent on the time factor of interaction with the insulin receptor, prolonged residence time as conclude from the incidence

of [B10-Asp] insulin would be dissociated with stimulation of tissue proliferation, and could have an effect on tumor progression (Prager and Melmed 1993, LeRoith 1994, DeMeys 1995b, Strobl et al. 1995).

The assessment of IGF-I receptor affinity and its that the physiological role becomes even more complicated with the unresolved role of insulin/IGF-I hybrid receptors (Soos et al. 1993, Takata and Kobayashi 1994), which may also have a role in the microvascular and macrovascular complications of diabetes (Li et al. 2005). At present time, there is no evidence for a pathophysiological role of slightly increased IGF-I receptor affinity in the presence of physiological residence time at the receptor, with dissociation rates similar to human insulin. Investigation of insulin glargine has shown that there is no effect on proliferation of benign and malignant mammary cell lines (Staiger et al. 2005), and no effect on tumor formation in rodent carcinogenicity studies (Stammberger et al. 2002). There is also no evidence for any proliferative effect of the clinically used fast acting insulin analogs in animal toxicology studies (EPARs for insulin lispro, insulin aspart, and insulin glulisine). Investigation of mammary gland tissue for proliferation markers after a six-month and twelve-months treatment in rats has shown no proliferative effect (Stammberger et al. 2006).

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K.9.5

Signaling via IGF-1 Receptor

The insulin receptor (IR) and the insulin-like growth factor I receptor (IGF-IR) belong to the same subfamily of receptor tyrosine kinases with two extracellular α -subunits and two transmembrane β -subunits. They share a high similarity of structure and intracellular signalling events (Dupont and Leroith 2002). However, the IR and the IGF-IR mediate different effects on metabolism, cell proliferation, apoptosis and differentiation. Although some of the variation can be attributed to a different tissue distribution or subcellu-

lar localization, it can also be explained by structural differences in the β -subunit, which may result in activation of specific substrates and signal pathways.

The presence and formation of hybrid receptors, heterodimers composed of the subunits of the insulin receptor and IGF-I receptor has been documented both in normal tissue and in tumor tissue samples (Salzman et al. 1984, Schumacher et al. 1991, Soos et al. 1993, Siddle et al. 1994). It has been proposed that signaling in tumor tissue may proceed via such hybrid receptors.

In cells expressing the insulin receptor isoform A (IRA) and the insulin like growth factor-I receptor (IGF1R), the presence of hybrid receptors, constituted of an alphabeta-IRA chain associated with an alphabeta-IGF1R chain, has been demonstrated. These heterodimers are found in normal cells, and also appear to play crucial roles in a number of cancers (Belfiore et al. 1999, Frasca et al. 2003, Pandini et al. 1999). However, they remain difficult to study, due to the concomitant presence of IRA and IGF1R homodimers. Using bioluminescence resonance energy transfer (BRET), Siddle and Blanquart et al. (2005) have developed assays to specifically monitor the activation state of IRA/IGF1R hybrids, both *in vitro* and in living cells. In the first assay, only hybrid receptors were BRET competent. In the second assay, the activation state of IRA/IGF1R hybrids was monitored in real time, in living cells. In hybrid receptors, trans-phosphorylation of the kinase-dead alpha/beta-Rluc moiety by the wild-type alpha/beta moiety induced the recruitment of YFP-PTP1B-D181A-Cter, resulting in a hybrid-specific ligand-induced BRET signal. Both methods allow monitoring of the activity of IRA/IGF1R hybrid receptor and could be used to detect molecules of therapeutic interest for the treatment of cancer (Blanquart et al. 2005).

CRITICAL ASSESSMENT OF THE METHOD

There are presently a number of methods applicable for the detection of insulin/IGF-I hybrid receptors, and their state of activation. It will be important to investigate insulin analogs with reference to their effects on hybrid receptors, and signaling in normal tissue (Lampshire and Lienhard 1992) as well as in tumor tissue. There are observations of adaptive changes in the formation of insulin/IGF-I hybrid receptors in type 2 diabetic patients, attributed to chronic hyperinsulinaemia (Mosthaf et al. 1991, Federici et al. 1998a, 1998b), as well as alterations of the hybrid receptor ratio in tumor as such as thyroid cancer (Belfiore et al. 1999), and breast cancer (Pandini et al. 1999, Frasca et al. 2003).

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K.9.6

Mitogenic Activity

GENERAL CONSIDERATIONS

The determination of mitogenic activity on benign and malignant cell lines is a preliminary approach which has been proposed in the points-to-consider document of the EMEA safety working party. Enhanced cell proliferation may be the result of signaling via the insulin receptor at near physiological concentrations, via the IGF-I receptor at supraphysiological concentrations, and presumably also related to the presence of hybrid receptors which are preferentially stimulated by IGF-I (Li et al. 2005, Slaaby et al. 2006)

PURPOSE AND RATIONALE

The tests for mitogenic activity are considered to be in early toxicology procedure which provides guidance about the subsequent toxicological evaluation to be performed. In case of enhanced mitogenicity found with the particular cell line, the six-month or 12 month toxicology studies may include histological investigation of specific organ tissues, together with proliferation markers in these tissues (as an example, referred to Stammberger et al. 2006 for investigation of mammary gland tissue in rats).

PROCEDURE

The procedure by Sliker et al. (1996) is based on stimulation of human mammary epithelial cells (HMEC). Mitogenicity was assessed by measuring insulin analog-stimulated growth of human mammary epithelial cells (HMEC) in culture. HMEC were obtained from Clonetics Corporation (San Diego, Calif., USA) at passage 7 and were expanded and frozen at passage 8. A fresh ampule was used for each experiment so that cells were not grown beyond passage 9. Cells were maintained in MCDB 170 medium containing bovine insulin (5 mg/ml), recombinant human epidermal growth factor (10 ng/ml), hydrocortisone (0.5 mg/ml), bovine pituitary extract (50 mg/ml)

and gentamycin/amphotericin B. For a growth experiment, cells were plated in 96-well trays at a density of 12500 cells/cm² in the above-medium modified as follows: 0.1% BSA was added and 5 mg/ml bovine insulin was substituted by a graded dose of human insulin or analog from 0 to 1000 nmol/l final concentration. Trays were incubated for 72 h and the cells were counted by Coulter counter (Coulter Electronics, Hialeah, Fla., USA) after trypsinization. Typically, the maximal growth response was between 3- and 4-fold stimulation over basal and did not differ between analogs. Response data were normalized to between 0 and 100% response equal to 100' (response at dose X – response at zero dose) divided by (response at maximal dose – response at zero dose). Dose-response data were fit by non-linear regression employing JMP (SAS Institute, Inc., Cary, N.C., USA).

The method by Trueb and Froesch in human osteosarcoma cells (SAOS-B10) for determination of the binding of insulin analogs to the IGF receptor, and proliferative activity was developed in 1995 and subsequently applied by Kurtzhals et al. (2000). Human osteosarcoma cells (B10, a subline of Saos-2, supplied by S.B. and G.A. Rodan, West Point, Pennsylvania) are used because they exhibit a high rate of proliferation and high number of IGF-1 receptors on the cell surface. From competition of 125-I-insulin by unlabeled human insulin, a number of only 740 insulin binding sites per cell has been calculated whereas 21,000 or 39,000 IGF-1 binding sites per cell were determined in two separate experiments. IGF-1 receptor binding is measured (Competition with 125-I IGF-I). The dilution of the stock solutions of the various analogs and natural insulins are performed with HBB/1% HSA (HBB: 118 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 8.8 mM D-Glucose, 100 mM Hepes, pH 8.0). In the studies by Froesch, possible concentration of the stock dilution was 5.5–10⁻⁸ M for insulin glargine and the related Arg(B31)Arg(B32) insulin due to a lower solubility of both substances at neutral pH.

Therefore, the maximum final concentration in the competition experiment was limited to 5.10⁻⁸ M for these analogs (10⁻⁸ M for human insulin and the animal insulins). The limitations of preparing stock solutions for insulin analogs with a marked change in isoelectric point should be considered in the experimental design. In another series, initial stock solutions with a standard concentration of 5.5 + 10⁻⁸ M were used, again limiting the final maximum concentration to 5.10⁻⁸ M for the insulin analogs in the competition experiment.

Osteosarcoma cells (BIO, passage 27, 125,000 or 150,000 per well) were seeded in 24-well tissue cul-

ture plates and kept overnight at 37°C in DMEM/F12 (1:1) with the addition of 10% fetal calf serum. For de-induction the medium was changed to 500 µl serum-free medium for 4 hours, then the cells were incubated with 200 µl HBB/1% HSA-[125-I]-IGF-1 tracer (0.03 pick/well = 52,000 cam, 8.1×10^{-13} M final concentration) + 20 µl insulin analog or reference in the respective dilution overnight at 4°C. Plates are washed three times with cold PBS and dried at room temperature. Then 200 µl 2 N NaOH are added to each well for 1 hour at room temperature. The lysed cells are transferred to scintillation vials (each well washed with additional 200 µl NaOH) counted for bound radioactivity with a gamma-counter (triplicate measurements).

Mitogenic activity was tested by thymidine incorporation. The dilution of the stock solutions of the various analogs and natural insulins was performed with the basic medium (F12/1 g BSA). The concentration range used was between 10(-11) and 10(-6) M. Cells (50,000) were seeded in 24-well tissue culture plates and kept overnight in DMEM/F12 (1:1) with the addition of 10% fetal calf serum at 37°C. The medium was changed to DMEM/F12 1:1 with 5% fetal calf serum for a second overnight incubation period, followed by F12/1 g BSA/L for 30 minutes. The medium was then changed to 400 µl (200 µl in another study) F12/1 BSA + 40 µl (20 µl in another study) insulin analog or reference compound in the respective dilution. Cells were kept at 37°C for 17 hours, then 30 µl (20 µl in another study) 3H-methylthymidine was added (0.3 pCi/well). After 3 to 4 hours at 37°C the medium was aspirated, plates were washed three times in cold PBS, and 500 µl 10% TCA added to each well for 30 minutes at 4°C. After aspiration, wells are washed twice with 10% TCA and dried at room temperature (30 minutes). Then 500 µl 1 N KOH is added to each well for 1 hour at room temperature. 250 µl are removed from each well for liquid scintillation counting (gamma-counter, triplicate counting) after mixing the sample first with 250 µl 1N-HCl and second with 5 ml scintillation fluid.

The method for mitogenic potency described by Kurtzhals et al (2000) is based on thymidine incorporation of the SAOS-B10 line with predominant IGF-I receptors and very few insulin receptors, previously used as SAOS cells extensively for research on chondrocytes and bone formation. The human osteosarcoma cell line Saos/B10 (20), a subclone of Saos-2 (ATCC HTB-85), was used by Kurtzhals. B10 cells express ~30,000 IGF-I receptors and <1,000 insulin receptors per cell. Cells were cultured in DMEM/F12 supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin. During the entire experiment, cells

were grown and kept at 37°C in a humidified atmosphere with 5% CO₂. For experiments, cells were trypsinized, plated in 24-well dishes at ~75–100 3 10³ cells per well, and cultured for 16 h. Growth medium was then removed and replaced with test buffer (DMEM/F12 without FCS but with 0.5% BSA), and the cells were starved for 4 h. Then, the medium was withdrawn, and cells were incubated with test buffer containing 0.8 pmol/l to 0.8 mmol/l insulin analog for 16 h at 37°C before addition of [3H]methyl-thymidine. [3H]thymidine incorporation was allowed to take place for 4 h at 37°C. The cells were then washed 3 times with ice-cold phosphate-buffered saline and lysed in 10% trichloroacetic acid for 15 min under constant gentle rocking at 4°C. The soluble free [3H]thymidine was removed by 3 consecutive washes in ice-cold 10% trichloroacetic acid. The high molecular weight part of the cell lysates was resolubilized in 1 mol/l KOH and neutralized with an equal volume of 1 mol/l HCl, and DNA-incorporated [³H]thymidine was measured in a b-counter. Each concentration was assayed in triplicate for each experiment. The experiments were repeated 3 times. To compare the mitogenic potential of the insulin analogs with that of human insulin, log-dose response profiles were generated, and EC₅₀ values were estimated using a 4-parameter logistic function (19). Relative mitogenic potency was calculated as the ratio between the estimated EC₅₀ values (EC_{50,HI}/EC_{50,analog}), assuming parallelism of the log-dose response profiles.

The relevance of these mitogenicity estimates in a special cell line with predominant IGF-I receptors has been challenged, in a particular because insulin glargine, which showed slightly enhanced mitogenic activity in this test dissociate is rapidly from the insulin receptor (similar to human insulin), and does not stimulate the IGF-I receptor at therapeutic concentrations in humans, because it's affinity for the IGF receptor over the therapeutic dose range is much lower than that of endogenous IGF-I at physiological concentrations.

MODIFICATIONS OF THE METHOD

These tests may be performed on cell lines in vitro, or on tissue specimens ex-vivo.

CRITICAL ASSESSMENT OF THE METHOD

Mitogenicity estimates our guidance for subsequent evaluation of tissues in animal toxicology studies.

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K.9.7

Insulin and IGF-1 Assays

GENERAL CONSIDERATIONS

Methods for the quantitation the determination of plasma concentrations of insulin, IGF-I and the IGF binding proteins (not considered here in detail) have been developed based on diverse analytical principles such as radioimmunoassay, enzyme-linked assays, chemiluminescence assays and other principles. Their particular application is to the measurement of concentration changes of insulin, IGF-I and binding proteins during repeated dose treatment of animals, and of course the main application in clinical chemistry is to the evaluation of type 2 diabetes, and growth disorders in children.

PURPOSE AND RATIONALE

The main purpose is to establish a relation of pharmacokinetics and pharmacodynamics, namely in states of insulin resistance (increased insulin concentrations) and during the treatment of diabetes (from decompensation before treatment to established glycemic control).

MODIFICATIONS OF THE METHOD

Specific methods for determination have been established for insulin analogs, for example aspart insulin (Andresen et al. 2000), lispro insulin (Bowsher et al. 1999; Cao et al. 2001), and some of these assays are available commercially (e. g., Linco assay for lispro insulin). For other insulin analogs, methods have been adapted for pharmacokinetic determination (e. g., Mudaliar et al. 2000; Kuerzel et al. 2003 for insulin glargine). There are many sensitive assays for animal insulins and for human insulin, which can be adapted for animal experimentation (Temple et al. 1992; Clark and Hales 1994), the insulin assays have been particularly useful in establishing physiological and pathological secretory patterns (Polonsky et al. 1988a, 1988b). Many of the studies have been extended and supplemented by the measurement of human or animal C-peptide in order to assess endogenous insulin production, and suppression of endogenous insulin during treatment (Ashby and Frier 1981; Bonser and Garcia-Webb 1981; Tillil et al. 1988). Measurement of excretion of C-peptide in the urine is also a helpful method of evaluating secretion rates.

Concerning the methods for determination of IGF-I, many methods were developed for pediatric applications and subsequently their use has been extended to other questions (Teale and Marks 1986; Quarmby et al. 1988; Tchao et al. 1995; Moses et al. 1996; Frystyk et al. 1999; Juul 2003; Elmlinger et al. 2004, 2005; Khosravi et al. 2005; Yakar et al. 2005). Methods for the determination of IGF binding proteins have been reviewed (Rutanen and Pekonen 1991), and several options are now available. In this context, the classical assays for IGF-I activity also need to be mentioned but no longer have practical relevance for the evaluation of the effects of insulin analogs (Zapf 1998).

In the context of measuring circulating concentrations, it may also be helpful to measure of the adaptation of receptor concentrations in tissues by using the appropriate insulin and IGF receptor assays *ex vivo* (Pedersen 1983; Taylor 1984; Johansson and Arnqvist 2006).

CRITICAL ASSESSMENT OF THE METHOD

Selection of the appropriate methods for the measurement of insulin, C-peptide, IGF-I and IGF binding proteins is facilitated by a number of options, based on laboratory developed methods and an increasing number of commercially available assays which have been extensively validated.

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K.9.8**Assessment of Metabolic-Mitogenic Ratio In Vitro****GENERAL CONSIDERATIONS**

When structure activity studies are performed with insulin analogs, it is useful to determine the relative contributions of the hypoglycemic activity (metabolic), and the proliferative activity (mitogenic). Both activities are intrinsic to the insulin molecule, it is important to realise that they are activated at vastly different concentrations in vitro, and that the wide concentration range for testing is permissible in such studies. The metabolic-mitogenic ratio needs to be supplemented by data on the receptor association/dissociation, because experimental evidence for the [B10-Asp] insulin with enhanced mitogenic activity and causing tumor stimulation in rodent toxicology studies indicates that prolonged residence time and decreased rate of dissociation from the insulin receptor may be the critical factor. As a consequence, this information is critical a required for a new insulin analogs. When additional information about enhanced affinity for the IGF-I receptor is obtained, mitogenicity may be tested by methods described here in K9.6 and needs to be followed up by studies on mammary epithelial cells, when available also on other benign and malignant tissue derived cell lines, and ultimately by assessment of carcinogenic potential in animals.

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K.9.9**Assessment of Hypoglycemic Activity In Vivo****GENERAL CONSIDERATIONS**

Methods for the assay of hypoglycemic activity in animals are reviewed in section K.3 and will be discussed here only with reference to the application and structure activity studies with insulin analogs. For the two classes of insulin analogs of clinical interest, a different experimental design is needed.

Tests by intravenous injection indicate the intrinsic activity, human insulin may be used as the comparator (Lin et al. 1999).

The time-action profiles of fast acting insulins are tested by subcutaneous injection in rats, rabbits or dogs, with regular human insulin as the reference preparation. The drug concentration of test insulin and reference human insulin needs to be identical, for meaningful comparisons. When dilution is compared, they should have the same insulin concentration. The preferred test is by using the under eluted insulin analog at the concentration equivalent to U-100. The test interval depends on the insulin dose selected, for fast acting insulins test periods of four to six hours are considered sufficient.

The time-action profiles of long acting insulin analogs are tested by subcutaneous injection preferably in dogs or pigs, as a preliminary procedure they may also be tested in rabbits. Human NPH insulin is used as the comparator. The preferred test concentrations are those equivalent to U-100 insulin. The zinc content of formulations is often of interest and will be tested at increasing zinc content, keeping the insulin drug concentration constant.

PURPOSE AND RATIONALE

The biological activity predicted by in vitro studies needs to be confirmed in animals, there may be considerable differences between the in vitro result and the *in vivo* activity due to the pharmacokinetics of the selected drug candidate. The first stage is always testing by intravenous injection to enable immediate contact with the insulin receptor.

K.9.9.1**Depot Activity of Insulin Analogs in Rabbits****PROCEDURE**

This test by subcutaneous injection is suitable for the evaluation of depot activity of long acting insulins and insulin depot preparations. Adult male rabbits body weight 2,5–3,5 kg are suitable for the test, the animals

may be used repeatedly when treatment free intervals of at least two weeks (recovery periods) are inserted after each treatment day. In each group, eight to 10 Rabbits are treated on the test day. Insulin analogs and insulin depot preparations are compared for reference. The insulin analogs (long acting) or depot preparations of human insulin as reference compounds (NPH insulin) are injected subcutaneously for example at a dose 0.3 IU/kg by microliter syringes, using the undiluted original concentration (straight concentrations) equivalent to U-100 insulin.

Blood samples for glucose determination are obtained from an ear vein immediately before treatment, and then hourly up to 8 hours after subcutaneous injection. The blood glucose may be determined by enzymatic assay, for example in 10 µl of native blood using a commercial test kit.

Evaluation of blood glucose data is was performed descriptively and by explorative statistics. For descriptive evaluation of time-action blood glucose profiles (time point and extent of maximum decrease, centroid time, duration/maximal decrease ratio and total area of blood glucose decrease are used.

In this experimental design, the concentration of the insulins and the composition may be varied, for example by changing the zinc content. To test statistically for dependency on insulin concentration or zinc content of the formulation, the drug concentration-dependency and/or the zinc concentration-dependency of two or more groups is compared using exact one-sided Wilcoxon tests (Hollander and Wolfe 1973, Streitberg and Röhmel 1987).

The test parameters are total peak area and ratio of duration to maximal decrease, accepting an increase within each of these parameters as the relevant test direction for increasing concentrations of drug (insulin) or zinc content (formulation). Each test is performed separately at a significance level of 5 %.

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MODIFICATIONS OF THE METHOD

The principal modifications of this method are the selection of insulin concentrations to be tested. It is established that depot activity depends on local concen-

tration at the injection site, therefore it is advisable to inject smaller volumes by microsyringe, rather than use dilution is of the original concentration to be evaluated.

CRITICAL ASSESSMENT OF THE METHOD

Testing of depot activity in rabbits is an earlier option, but not as trustworthy as extended testing for depot activity in dogs or pigs.

K.9.9.2

Depot Activity of Insulin Analogs in Fasted Dogs

GENERAL CONSIDERATIONS

The time action profile of long acting insulin analogs after subcutaneous injection varies considerably depending on the species used for testing. In general, proof of depot activity cannot be obtained in mice and rats, is difficult to obtain in rabbits unless high dose is a tested, and is readily obtained by testing in in dogs or pigs.

PURPOSE AND RATIONALE

The test procedure in dogs is based on experience with human NPH insulin and with insulin zinc suspensions (intermediate acting insulins). It has shown to be very reliable in the preclinical evaluation of insulin glargine (Seipke et al. 2000),

PROCEDURE

Adult male beagle dogs are used for this study, feed is withheld for 17 hours. Groups of dogs (six to 10 animals) are treated with the insulin to be tested for depot activity. Human regular insulin (soluble) and/or human NPH insulin are used as the reference compounds. Tests may be performed sequentially, in parallel or by cross over. Insulin preparations are injected subcutaneously at the flank of the animal, alternating between the right and left flank. Blood samples for glucose determination are obtained by leg vein puncture, immediately before treatment and hourly up to 10 hours after treatment, additional samples may be obtained 12, 14 and 16 hours after administration. Blood glucose is determined enzymatically, for example in 10 µl native blood using the Glucoquant test kit (Boehringer Mannheim) with the EPOS Analyzer 5060 (Eppendorf). For evaluation of the glucose data, means and standard error of the mean at each measuring point of the raw data [or using data converted to percent of the initial values] were used for tabular and/ or graphical representation of the time response curves.

For statistical evaluation of the depot activity, centroid times are calculated and, for evaluation of the blood glucose decreasing activity, the area of blood glucose decrease for each group is calculated. In both cases, a two-way ANOVA is performed at a significance level of 5%.

The mean blood glucose decrease was calculated by the trapezoidal rule and the centroid time (C_t) by

$$C_t = \frac{\sum_{i=1}^n (T_i * F_i)}{\sum_{i=1}^n F_i} \quad \text{where} \quad T_i = \frac{t_{i+1} + t_i}{2} \quad \text{and}$$

$$F_i = \frac{(t_{i+1} - t_i)(c_{i+1} - c_i)}{2}$$

t_i = times of measurement ($i = 1, \dots, n$),

c_0 = base line glucose concentration and

c_i = glucose concentration at measurement time t_i .

Calculation of the centroid time and of the areas of blood glucose decrease is performed with the raw data. The point of onset and endpoint of the blood glucose decrease are defined by intersection of the blood glucose curve with the baseline in the descending or ascending phase, respectively.

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K.9.10

Mitogenic Risk and Safety Evaluation In Vivo

GENERAL CONSIDERATIONS

An intense discussion was raised by the findings that one fast acting insulin analog [B10-Asp] insulin showed unexpected toxicity on proliferation and tumor development of rat mammary glands. This finding was attributed tentatively to mitogenic signaling via the insulin receptor, and/or mitogenic signaling via the IGF-I receptor, based on the finding that on the insulin receptor residence time was prolonged and the rate of dissociation was much lower than found with

human insulin, and that affinity for the IGF-I receptor was enhanced. This compound has therefore become the reference standard for the assessment of enhanced mitogenicity and its effect in toxicology. The preclinical evaluation of insulin analogs for the risk of enhanced mitogenicity therefore includes determination of the metabolic and mitogenic in vitro characteristics, by affinity for the insulin receptor and rate of association/dissociation, affinity for the IGF-I receptor, evaluation of enhanced mitogenic signaling e. g. by thymidine incorporation into proliferating cells, and details of signaling via the insulin receptor and IGF-I receptor (e. g. tyrosin phosphorylation, phosphorylation of receptor substrates).

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Chapter L

Anti-obesity Activity¹

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L.1 Methods to Induce Experimental Obesity

L.1.0.1 General Considerations

Influence of the central nervous system, in particular of the hypothalamus, on development of obesity has been suspected since the early clinical observations of Babinski (1900) and Fröhlich (1901), Biedl 1916). Experiments reported by Smith (1927, 1930) showed that injections of chromic acid into the suprasellar region of rats with lesion of the hypothalamus induced obesity in rats (Bomskov 1939). Hetherington and Ranson (1939) found that electrolytic lesions, restricted to the ventromedial region of the hypothalamus, could be associated with the development of obesity.

A virally induced obesity syndrome in mice was described by Lyons et al. (1982).

Chan (1995) gave a review on β -cell stimulus-secretion coupling defects in rodent models of obesity.

Leiter and Herberg (1997) reviewed the advances in understanding the molecular bases for monogenic obe-

¹Reviewed by M. Bickel, contributions by A.W. Herling

sity mutations capable of producing obesity-induced diabetes, or diabetes in mice.

Astrup and Lundsgaard (1998) discussed pharmacological mechanisms of anti-obesity drugs.

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and 19% water. Body weight and food intakes are measured, and diet replaced, every 3 to 4 days.

Three months from the start of the experiment, the rats are sacrificed by decapitation for determination of adipose tissue cell size and number, carcass composition and plasma lipids, and hormone and glucose levels.

The dorsal subcutaneous (inguinal) pad, the retroperitoneal pad, and one epididymal fat pad are sampled for determination of lipid content by the method of Folch et al. (1957). The method consists of homogenizing the tissue with a 2:1 chloroform-methanol mixture and washing the extract by addition to it of 0.2 its volume of water. The resulting mixture separates into two phases. The lower phase is the total pure lipid extract. Cell number in each pad is determined by the osmium fixation method of Hirsch and Gallian (1968) using a Coulter counter. Total epididymal pad weights are based on doubling the weight of the individual pads sampled.

EVALUATION

Intergroup comparisons are made with the use of a two-tailed Student's *t*-test.

MODIFICATIONS OF THE METHOD

Scalfani and Springer (1976) induced obesity in adult female rats by adding a variety of supermarket foods to lab chow (“cafeteria diet”). In behavioral tests, the authors found similarities to hypothalamic and human obesity syndromes.

Rothwell et al. (1982) compared the effects of feeding a cafeteria diet on energy balance and diet-induced thermogenesis in four strains of rats.

Stock and Rothwell (1979) discussed the influence of various forms of feeding, high-fat diets, insulin injections, tube-feeding, and cafeteria feeding on energy balance in laboratory animals.

Rolls et al. (1980) found persistent obesity in rats following a period of consumption of a mixed, high energy diet. When the high energy foods were withdrawn after 90 days and just chow was available, the obese rats maintained their elevated body weights.

Hill et al. (1992) studied the influence of amount and composition of dietary fat on development of obesity in rats. Adult male Wistar rats were fed high fat (HF; 60% of calories) or low fat (LF; 20% of calories) diets for 28 weeks. Half of the rats in each condition received diets with saturated fat (lard) or with polyunsaturated fat (corn oil). There was some indication that unsaturated fat diets were associated with greater ac-

L.1.0.2

Food-Induced Obesity

PURPOSE AND RATIONALE

Obesity can be induced in rats by offering a diet containing corn oil and condensed milk.

PROCEDURE

Male Sprague Dawley rats are housed in individual wire-bottom suspended cages in rooms maintained at 22–23°C with 12 h light-dark cycles. At the age of 6 months (body weight about 450 g) the animals are divided in 2 groups: group I is fed ordinary Purina Rodent Chow, group II a special diet containing Purina Rodent Chow, corn oil and condensed milk, resulting in a composition of 14.7% protein, 44.2% carbohydrate, 15.8% lipid, 2.5% fiber, 1.2% vitamin mixture,

cumulation of fat in subcutaneous tissue depots than saturated fat diets. The effects of the type of fat were less than those of the amount of dietary fat.

Wade and Gray (1979) reviewed the gonadal effects on food intake and adiposity in rats. Estradiol and testosterone decrease adiposity, while progesterone increases carcass fat content.

Thermogenesis induced by various diets has been discussed by Rothwell and Stock (1986).

Harris (1993) studied the impact of high- or low-fat cafeteria foods on nutrient intake and growth of rats consuming a diet containing 30% energy as fat.

Segues et al. (1994) studied long-term effects of cafeteria diet feeding on young female Wistar rats by comparing the circulating levels of glucose, lactate, glycerol, 3-hydroxybutyrate and urea, and liver glyco-

gen. Llado et al. (1997) studied fatty acid composition of brown adipose tissue in dietary obese rats. Long time exposure to a hypercaloric high-fat diet such as the cafeteria diet induced an important fatty acid accumulation in brown adipose tissue, mainly for the major saturated and monounsaturated fatty acids.

LeBlanc and Labrie (1997) investigated the role of palatability of the food in diet-induced thermogenesis.

Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats was reported by Levin et al. (1997).

Herberg et al. (1974) reported the effects of either a high-carbohydrate diet or a high-fat diet over prolonged periods in metabolically intact and in obese NZO mice.

West et al. (1992) evaluated the effects of a 7 week consumption of a diet containing 32.6% of kilocalories as fat (condensed milk) on body composition and energy intake in nine strains of inbred mice. Relative to Chow diet controls, the condensed milk diet significantly increased carcass lipid content in six strains (AKR/J, C57L/J, A/J, C3H/HeJ, DBA/2J, and C57BL/6J), but had no or a marginal effect on adiposity in 3 strains of mice (SJL/J, I/STN, and SWR/J).

West et al. (1994) studied the genetics of dietary obesity in AKR/J x RWR/J mice. Pups were weaned between the ages of 29 and 34 days onto Purina Chow ad libitum until they were switched to a high fat, condensed milk diet containing 32.6%, 15.0%, and 52.4% of kilocalories as fat, protein, and carbohydrate, respectively.

Strain-specific response to β_3 -adrenergic receptor agonist treatment of diet-induced obesity in mice was reported by Collins et al. (1997).

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L.1.0.3

Hypothalamic Obesity

PURPOSE AND RATIONALE

Hyperphagia in rats has been reported after hypothalamic lesions (Liu and Yin 1974). Surgical techniques were described by Leibowitz et al. (1981), Vander Tuig et al. (1985).

PROCEDURE

Female Sprague Dawley rats, weighing about 190 g body weight, receive during a 5–9 days initial period of adjustment a high-fat diet. They are then fasted overnight, anesthetized with 35 mg/kg pentobarbital sodium and additionally 1 mg atropine methyl nitrate intraperitoneally. Bilateral wire knife cuts or electrolytic lesions are stereotaxically positioned in the hypothalamus (David Kopf Instruments, CA). With the incision bar positioned at –3.0 mm, parasagittal wire knife cuts are placed between the medial and lateral hypothalamus using a retractable wire knife according to Gold et al. (1973). The cuts are made 1.0 mm lateral to the midline and extended from 8.5 to 5.5 mm anterior to the ear bars and from the base of the brain dorsally 3.0 mm.

To produce electrolytic lesions in the ventromedial hypothalamus, the incision bar is positioned at +5.0 mm and a stainless steel electrode, insulated except for 0.5 mm at the tip, is lowered 0.6 mm lateral to the midline and 5.8 mm anterior to the ear bars. With the tip of the electrode 0.7 mm above the base of the brain, lesions are made by passing 2.0 mA of anodal current for 20 s to a rectal electrode.

Sham-operated rats serve as controls. Separate rats that are fasted overnight are killed at the time of surgery to provide data on initial body composition.

Histological verification of placement of knife cuts and lesions is made in brains fixed in 10% buffered formaldehyde solution and embedded in paraffin. Serial sections through the hypothalamic area of the brain are examined histologically.

EVALUATION

Not only food consumption and increase of body weight can be determined after hypothalamic lesions, but also brown adipose tissue enzymes and guanosine diphosphate binding to brown adipose tissue mitochondria and noradrenaline turnover in various organs.

MODIFICATIONS OF THE METHOD

A survey on hypothalamic and genetic obesity in experimental animals was given by Bray and York (1979).

Caloric compensation to gastric loads in rats with hypothalamic hyperphagia was reported by Liu and Yin (1974).

Sclafani and Aravich (1983) adapted adult female Sprague Dawley rats to a macronutrient self-selection regimen which allowed them ad libitum access to separate sources of protein, carbohydrate, and fat. The rats were then given either ventromedial hypothalamic lesions, paraventricular hypothalamic lesions, parasagittal knife cuts through medial hypothalamus or sham lesions. Following surgery, all lesioned rats overate and became obese as compared to sham operated controls. The group with parasagittal knife cuts through medial hypothalamus gained more weight than the groups with ventromedial hypothalamic lesions and paraventricular hypothalamic lesions.

Enhanced expression of rat obese (ob) gene in adipose tissue of ventromedial hypothalamus (VMH)-lesioned rats was described by Funahashi et al. (1995).

Elmqvist et al. (1999) discussed the role of leptin and other peptide hormones in different areas of the hypothalamus in controlling food intake and body weight.

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L.1.0.4

Goldthioglucose-Induced Obesity

PURPOSE AND RATIONALE

Intraperitoneal or intramuscular injection of goldthioglucose induces obesity in mice. The effect is related to destruction of hypothalamic and extra-hypothalamic areas of the brain (Perry and Liebelt 1961).

PROCEDURE

Swiss albino mice of either sex are fed with commercial mouse chow (Altromin R) ad libitum. At the age of 6 weeks, the animals receive a single intraperitoneal injection of 30–40 mg/kg goldthioglucose.

EVALUATION

Food intake is registered for 2 weeks and body weight for a period of 3 months and compared with untreated controls.

MODIFICATIONS OF THE METHOD

Debons et al. (1962, 1968, 1977) administered 800 mg/kg goldthioglucose by a single intraperitoneal injection to female CBA mice.

Obesity was induced in rats by implants of goldthioglucose in the hypothalamus (Smith and Britt 1971; Smith 1972).

Several other compounds produce obesity concomitantly with brain lesions.

Single intraperitoneal injection of bipiperidyl mustard (N,N'-bis-[β -chloroethyl]-4,4'-bipiperidine) in doses between 5 to 50 mg/kg induced obesity in mice (Rutman et al. 1966). Brain lesions and obesity by bipiperidyl mustard could also be produced in rats (Laughton and Powley 1981).

Massive and persistent obesity was induced in mice by a single intracerebral injection with 4-nitroquinoline 1-oxide (Mizutani 1977).

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L.1.0.5

Monosodium Glutamate-Induced Obesity

PURPOSE AND RATIONALE

Adiposity can be induced in mice by repeated subcutaneous injections of monosodium-L-glutamate at an early stage of life (Olney 1969).

PROCEDURE

Male Charles River mice are treated immediately after birth with daily subcutaneous injections of 2 g/kg monosodium-L-glutamate for 5 consecutive days. Control mice are treated with physiological saline. The animals are weaned at 3 weeks of age, housed under controlled temperature and artificial light/dark cycle and provided with commercial powdered chow and tap water ad libitum.

EVALUATION

Food consumption and weight gain is measured at weekly intervals.

MODIFICATIONS OF THE METHOD

Tokuyama and Himms-Hagen (1986) studied brown adipose tissue thermogenesis, torpor, and obesity in glutamate-treated C57B1/6J mice offered either normal chow or a cafeteria diet and found that the high metabolic efficiency and obesity of the glutamate-obese animals are principally a consequence of their

maintenance of a hypothermic torpid state for more than 50% of the time.

Seress (1982) injected young albino **rats** with either single or repeated doses of 0.1–6.0 mg/g body weight of monosodium L-glutamate between the ages of 2 and 40 days. The smallest, single effective dose was 0.25 mg/g body weight administered during the first week of life. The sensitivity to monosodium L-glutamate decreased with age. In adult rats an 80–90% loss of neurons in the anterior part of the arcuate nucleus was found.

Remke et al. (1988) reported on development of hypothalamic obesity after subcutaneous administration of monosodium glutamate to neonate rats.

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L.2

Genetically Obese Animals

L.2.0.1

General Considerations

Obesity and diabetes are syndromes quite often linked in patients (maturity onset diabetes) and hereditary animal models. Hunt et al. (1976) described animal

models of diabetes and obesity including the PBB-Ld mouse. A survey on hypothalamic and genetic obesity in experimental animals was given by Bray and York (1979). The inheritance of obesity in animal models of obesity was discussed by Festing (1979). Cawthorne (1979) discussed the use of animal models in the detection and evaluation of compounds for the treatment of obesity. The regulation of body weight in animals by leptin was reviewed by Friedman and Halaas (1998).

Symptoms of diabetes and obesity are overlapping in many animal models (see also K.2: Genetically diabetic animals).

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L.2.0.2

Spontaneously Obese Rats

The occurrence of spontaneous obesity has been reported in several strains of rats:

WBN/KOB RAT

Spontaneous hyperglycemia, glucosuria and glucose intolerance have been observed in aged males of an inbred Wistar strain, named the WBN/Kob rat (Nakama et al. 1985; Tsuchitani et al. 1985; Koizumi 1989). These animals exhibit impaired glucose tolerance and glucosuria at 21 weeks of age. Obvious decreases in the number and size of islets are found already after 12 weeks of age. Fibrinous exudation and degeneration of pancreatic tissue are observed in the exocrine part, mainly around degenerated islets and pancreatic ducts in 16 weeks old males.

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Zucker-Fatty Rat

The Zucker-fatty rat is a classic model of hyperinsulinemic obesity. (Zucker 1965). Obesity is due to a simple autosomal recessive (*fa*) gene and develops at an early age. Obese Zucker rats manifest mild glucose intolerance, hyperinsulinemia, and peripheral insulin resistance similar to human NIDDM. However, their blood sugar level is usually normal throughout life (Bray 1977; Clark et al. 1983; McCaleb and Sredy 1992; Abadie et al. 1993; Alamzadeh et al. 1993; Kasim et al. 1993; Galante et al. 1994).

Truett et al. (1991) found evidence that the rat obesity gene fatty (*fa*) has homology with the mouse gene diabetes (*db*).

Triscari and Sullivan (1987) reported a normalizing effect of an inhibitor of thromboxane synthase on the hyperinsulinemic state of obese Zucker rats and diet-induced obese rats.

Rouru et al. (1993) described the effect of chronic treatment with a 5-HT₁ receptor agonist on food intake, weight gain, plasma insulin and neuropeptide Y mRNA expression in obese Zucker rats.

Santti et al. (1994) studied the potentiation of the anti-obesity effect of a β_3 -adrenoceptor agonist in obese Zucker rats by exercise.

Savontaus et al. (1997) investigated the anti-obesity effect of a imidazoline derivative in genetically obese (*fa/fa*) Zucker rats.

Zhang et al. (1996) reported downregulation of the expression of the *obese* gene by an antidiabetic thiazolidinedione in Zucker diabetic fatty rats and *db/db* mice.

Lynch et al. (1992) identified several adipocyte proteins, among them pyruvate decarboxylase contributing to the increased lipogenic capacity of young obese Zucker adipocytes.

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WDF/TA-FA RAT

The WDF/Ta-fa rat, commonly referred to as the Wistar fatty rat, is a genetically obese, hyperglycemic rat established by the transfer of the fatty (*fa*) gene from the Zucker rat to the Wistar Kyoto rat. (Ikeda et al. 1981; Kava et al. 1989; Velasquez et al. 1990). The Wistar fatty rat exhibits obesity, hyperinsulinemia, glucose intolerance, hyperlipidemia, and hyperphagia similar to Zucker rats being, however, more glucose intolerant and insulin resistant than Zucker rats. Hyperglycemia is usually not observed in females but can be induced by addition of sucrose to the diet.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activation of insulin receptor kinase by Pioglitazone in Wistar fatty rats (*fa/fa*).

Mazusaki et al. (1996) found an augmented expression of the *obese* (*ob*) gene during the process of obesity in genetically obese-hyperglycemic Wistar fatty (*fa/fa*) rats.

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OLETF Rat

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long-Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and named OLETF. The characteristic features of OLETF rats are: (1) late onset of hyperglycemia (after 18 weeks of age),

(2) a chronic course of disease, (3) mild obesity, (4) inheritance by males, (5) hyperplastic foci of pancreatic islets, and (6) renal complications (nodular lesions). The clinical and pathological features of disease in OLETF rats resemble those of human NIDDM.

Administration of diazoxide (0.2% in diet), an inhibitor of insulin secretion, to OLETF rats from the age of 4 to 12 weeks completely prevented the development of obesity and insulin resistance (Aizawa et al. 1995).

Ishida et al. (1995) found that insulin resistance preceded impaired insulin secretion in OLETF rats.

Umekawa et al. (1997) determined induction of uncoupling protein and activation of GLUT4 in OLETF rats after administration of a specific β_3 -adrenoceptor agonist.

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Obese SHR Rat

The strain of obese SHR rats was developed by Koletsky (1973, 1975) by mating a spontaneous hypertensive female rat of the Kyoto-Wistar strain with a normotensive Sprague Dawley male. After several generations of selective inbreeding, these obese SHR exhibited obesity, hypertension, and hyperlipidemia. In addition, some rats developed hyperglycemia and glucosuria associated with giant hyperplasia of pancreatic islets.

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JCR:LA-Corpulent Rat

Several substrains were developed from obese SHR rats, such as the JCR:LA-corpulent rat which exhibits a syndrome characterized by obesity, hypertriglyceridemia and hyperinsulinemia with impaired glucose tolerance and is susceptible to vascular arteriosclerotic lesions (Russell et al. 1986a, b, 1994).

Compared to fatty Zucker rats, the JCR:LA-corpulent rats have higher levels of the insulin releasing hormone gastric inhibitory polypeptide and higher insulin levels (Pederson et al. 1991).

Vydelingum et al. (1995) found an overexpression of the obese gene in the JCR:LA-corpulent rat.

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Growth Hormone-Deficient Dwarf Rat

Clark et al. (1996) described the obese growth hormone-deficient dwarf rat as a new model of obesity.

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L.2.0.3**Spontaneously Obese Mice****Yellow Obese ($A^Y A$) Mouse**

The yellow obese mouse is the only example of obesity inherited through a dominant gene and was described as early as 1883 by Lataste and in 1905 by Cuenot. It is located on chromosome 2 at linkage group 5, the agouti locus (Bateson 1903). Since the genes controlling obesity and the agouti coat colors are so closely linked, the obesity is associated with a change of pigmentation from black to yellow. Such an association allows the early identification of pre-obese mice as soon as the coat hair begins to grow.

Since the original description of the yellow ($A^y a$) mouse, a number of additional alleles have appeared at the agouti locus. The homozygous dominant yellow mutation (A^y/A^y) is lethal in utero (Robertson 1942; Eaton and Green 1962) with approximately 25% of any litter from $A^y a$ matings dying from an abnormal development after the trophoblast stage (Pedersen 1974).

Yellow ($A^y a$) mice develop a moderate form of obesity and diabetes. Increased body weight first appears at the time of puberty (8–12 wk) (Dickie and Wooley 1946; Carpenter and Mayer 1958), after which body weight increases slowly to reach values of approximately 40 g. In contrast to other forms of obesity, yellow mice are characterized by increased linear growth. Plasma insulin concentrations are increased and food is stored more efficiently than in lean mice (Dickerson and Gowan 1967). Food intake returns to normal in older $A^y a$ mice and the animals lose body weight (Hollifield and Parson 1957). The obesity may be exaggerated by being fed high-fat diets (Fenton and Chase 1951; Silberberg and Silberberg 1957; Carpenter and Mayer 1958). Food restriction may normalize body weight but the animals still remain obese (Fenton and Chase 1951; Hollifield and Parson 1957). Metabolic rate of $A^y a$ mice is depressed when related to body surface, although oxygen consumption per animal is identical to the homozygous recessive agouti (a/a) mouse (Bartke and Gorecki 1968).

Gill and Yen (1991) studied the effect of ciglitazone on endogenous plasma islet amyloid polypeptide

(amylin) and insulin sensitivity in obese-diabetic viable yellow mice (VY/Wfl-A^{vy/a}).

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KK-A^y Mouse

Iwatsuka et al. (1970) reported on yellow KK mice (also named KK-A^y mice), carrying the yellow obese gene (A^y). These mice develop marked adiposity and diabetic symptoms in comparison with their littermates, black KK mice. Blood glucose and circulating insulin levels as well as HbA_{1c} levels were increased progressively from 5 weeks of age. Degranulation and glycogen infiltration of B cells were followed by hypertrophy and central cavitation of islets. Lipogenesis by liver and adipose tissue were increased. Insulin sensitivity of adipose tissue was more remarkably reduced than in black KK mice to its complete loss at 16 weeks of age. Renal involvement is uniquely marked by early onset and rapid development of glomerular basement membrane thickening (Diani et al. 1987).

KK-A^y mice can be used to demonstrate the extra-pancreatic action of antidiabetic drugs, such as glimepiride, a novel sulfonylurea (Satoh et al. 1994).

Sohda et al. (1990) evaluated ciglitazone and a series of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazoli-

dinediones for hypoglycemic and hypolipemic activities in yellow KK mice.

Hofmann et al. (1992) evaluated the expression of the liver glucose transporter GLUT2 and the activity and the expression of phosphoenolpyruvate carboxykinase in the liver of obese KKA^y mice after treatment with the oral antidiabetic agent pioglitazone.

Yoshida et al. (1991) compared brown adipose tissue thermogenesis, resting metabolic rate, insulin receptors in adipocytes, and blood glucose and serum insulin levels during a glucose overloading test in yellow KK mice with C57B1 control mice after a β_3 -adrenoceptor agonist.

Yoshida et al. (1996) determined body weight, food intake, white adipose tissue weight, brown adipose tissue weight and its thermogenesis, noradrenaline turnover, blood glucose and serum insulin levels and GLUT4 in diabetic yellow KK mice compared with C57B1 mice after mazindol.

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OBESSE HYPERGLYCEMIC (OB/OB) MICE

Ingalls et al. (1950), Mayer et al. (1951), Bleisch et al. (1952) observed hereditary diabetes in genetically obese mice. The obese hyperglycemic mice were glycosuric, the non-fasting blood sugar levels were about 300 mg%, but neither ketonuria nor coma were observed. One of the most interesting features was insulin-resistance; doses as high as 400 IU/kg had little effect on blood sugar. The

serum insulin-like activity was high, the islands of Langerhans were hypertrophic, their insulin content was increased and the liver glycogen stores were decreased. Kidneys and other organs did not show pathological changes. Obviously, the diabetic condition of this and other strains of obese hyperglycemic mice is different from that of the human diabetic patient.

Reduced oxygen consumption has been noted as early as 10–18 days of age in *ob/ob* mice (Boissenaux et al. 1976; Trayhurn 1977).

Other strains or substrains of mice with obesity and hyperglycemia have been described by Dickie (1962), Westman (1968), Stein et al. (1970), Coleman and Hummel (1973), Herberg and Coleman (1977).

Strautz (1970) implanted *ob/ob* mice with Millipore diffusion chambers containing islets isolated from pancreas of normal littermates.

Trayhurn et al. (1977) found a thermogenic defect in pre-obese *ob/ob* mice. Rectal temperature of 17 days old pre-obese mice in response to an environmental temperature of 4°C fell much more than in lean controls.

Chlouverakis (1972) performed parabiotic experiments of obese-hyperglycemic mice (*ob/ob*) with lean littermates and determined body weight, glucose, serum insulin and triglycerides as well as insulin-sensitivity of diaphragm muscle and epididymal fat pad.

Parabiosis of obese (*ob/ob*) with diabetes (*db/db*) mice caused the obese partner to become hypoglycemic, to lose weight and to die of starvation, while no abnormal changes were observed in the diabetic partner (Coleman 1973).

Cresto et al. (1977) compared the rate of insulin degradation in normal and *inob/ob* mice.

Zhang et al. (1994) succeeded in positional cloning of the mouse *obese* gene and its human homologue.

Pelleymounter et al. (1995) investigated the effects of the *obese* gene product on body weight regulation in *ob/ob* mice. The OB protein was expressed in *E. coli* and purified to homogeneity as a 16-kilodalton monomer. Daily intraperitoneal injections of the recombinant OB protein to *ob/ob* mice lowered their body weight, percent body fat, food intake, and serum concentrations of glucose and insulin.

Halaas et al. (1995) reported that daily intraperitoneal injections of either mouse or human recombinant OB protein reduced the body weight of *ob/ob* mice but had no effect on *db/db* mice.

Campfield et al. (1995) found that peripheral and central administration of microgram doses of recombinant mouse OB protein reduced food intake and body

weight of *ob/ob* and diet-induced obese mice but not in *db/db* obese mice.

Trayhurn et al. (1996) studied the effects of fasting and refeeding on *ob* gene expression in white adipose tissue of lean and obese (*ob/ob*) mice using a 33-mer antisense oligonucleotide as a probe for the rapid chemiluminescence-based detection of *ob* mRNA.

Sterility defect in homozygous obese female mice could be corrected by treatment with the human recombinant OB protein leptin (Chehab et al. 1996).

Roupas et al. (1990) used isolated adipocytes from *ob/ob* mice to study the diabetogenic action of growth hormone.

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BL/6 Obese Mice

An obese mutation occurred in a noninbred stock (Ingalls et al. 1950) but was established later, and has been maintained, in the C5BL/6J (BL/6) strain. BL/6 obese mice are characterized by marked obesity, hyperphagia, transient hyperglycemia and markedly elevated plasma insulin concentrations associated with an increase in number and size of beta cells in the islets of Langerhans (Coleman and Hummel 1973; Genuth et al. 1971). The mutation is autosomal recessive and homozygous mutants of both sexes are infertile. Obese mutants are obtained by mating known heterozygotes. A primary metabolic disturbance in the adipocyte has been postulated since increased adipocyte size has been observed as early as 14 days of age well before any obesity or hyperinsulinemia are observed (Joosten and van der Kroon 1974).

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NZO Mouse

The New Zealand obese (NZO) mouse was first described in 1953 by Bielschowsky and Bielschowsky. The strain was developed by selective inbreeding of obese mice from a mixed colony, beginning from a pair of agouti mice, which also gave rise to the NZB black strain (Melez et al. 1980). NZO mice develop obesity, mild hyperglycemia, glucose intolerance, hyperinsulinemia, and insulin resistance. The adult NZO mouse normally attains a body weight of 50–70 g by 6–8 months, although weight gain continues slowly after this age (Crofford and Davis 1965; Herberg et al. 1970). Hyperglycemia and glucose intolerance increase continuously with advancing age of the animals.

Renal disease in NZO mice is seen by 6 months of age. NZO mice have a high prevalence of autoimmune disorders.

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Diabetes Obesity Syndrome in CBA/CA Mice

A spontaneous maturity onset diabetes obesity syndrome occurs in a small proportion (10–20%) of male CBA/CA mice. Inbreeding can increase the incidence to 80%. It occurs at 12–16 weeks of age, and is characterized by hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and an impaired glucose tolerance. The mice are also resistant to exogenous insulin. Female mice remain normal except

for a slight increase in serum insulin. The male obese diabetic mice have a normal life expectancy.

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FAT/FAT Mice

Fat mice carry an autosomal recessive mutation and display a range of abnormalities, including progressive adult onset obesity, hyperinsulinemia and infertility (Coleman and Eicher 1990). The mutant allele of *fat* was identified and shown to be a missense (serin → proline) mutation in carboxypeptidase E which abolishes enzyme activity in a variety of neuroendocrine tissues (Naggert et al. 1995). Carboxypeptidase E is required for both sorting and proteolytic processing of a variety of prohormones including proinsulin and POMC (Cool et al. 1997). As carboxypeptidase E is expressed in the CNS, defective processing of a variety of hypothalamic neuropeptides – such as POMC and MCH – may trigger obesity in these animals (Rovere et al. 1996).

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Tubby Mice

Tubby is an autosomal recessive mutation in mice (Coleman and Eicher 1990) which display a tripartite phenotype of blindness, deafness and maturity onset obesity. In response to weight gain, these mice

gradually increase their food intake in proportion to body weight and increase plasma insulin levels thereby maintaining normoglycemia. The progressive retinal degeneration in *tubby* mice results from apoptotic loss of photoreceptor cells, with abnormal electroretinograms detected as early as 3 weeks of age (Heckenlively et al. 1955). The mouse obesity gene *tubby* has been identified and characterized (Noben-Trauth et al. 1996; Kleyn et al. 1996).

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L.2.0.4

Transgenic ANIMALS

PURPOSE AND RATIONALE

Transgenic animals will offer a new approach to study development of obesity and therapeutic possibilities. Lowell et al. (1993) used a transgenic toxigene approach to create transgenic mice with primary deficiency of brown adipose tissue.

The potential for inserting new genetic material into mammals has produced numerous transgenic mice with increased or decreased quantities of body fat (Bray and Bouchard 1997).

Jensen et al. (1997) reported prevention of diet-induced obesity in transgenic mice overexpressing skeletal muscle lipoprotein lipase. The authors hypothesized that the potential to increase lipoprotein lipase activity in muscle by gene or drug delivery may prove an effective tool in preventing and/or treating obesity in humans.

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Lowell BB, Susulic VS, Haman A, Lawitts JA, Himms-Hagen J, Boyer BB, Kozak LP, Flier JS (1993) Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* 366:740–742

L.3 Assays of Anti-obesity Activity

L.3.1 Anorectic Activity

L.3.1.1 Food Consumption in Rats

PURPOSE AND RATIONALE

Food intake is measured in acute experiments in normal or obese rats. Additionally, in semichronic experiments body weight gain is recorded.

PROCEDURE

Female Zucker rats weighing 250–350 g are maintained under standard conditions (temperature, light-dark cycle, ground rodent pellet chow, tap water). Measurements of food intake begin on day –2. The food is offered in special dishes to reduce spillage. Food intake and body weight are measured daily between 8:00 and 9:00 A.M. At this time any spilled food from the collecting paper under the cage is gathered, air-dried if necessary, and weighed. Individual food intakes in grams are recorded. The tests compounds are either administered with the food or injected intraperitoneally. Groups of 5–10 animals are used for control or treatment with various doses of the test compound or the standard. Mazindol, 3 m/kg i.p. or 10 mg/kg p.o. can serve as standard. Treatment is continued for 7 days.

EVALUATION

Average food intake and body weight is recorded for each day. Average values of test drugs are compared statistically for each day with the control group. Results after oral administration have to be confirmed by parenteral route in order to exclude errors due to palatability.

MODIFICATIONS OF THE METHOD

Hull and Maher, Maher and Hull (1990) used male Sprague-Dawley rats placed on a mush diet composed of equal parts of ground rodent chow and of 4% nutrient agar solution. The agar-based chow allows a more accurate measurement of food intake and has been shown to be sufficient for maintaining normal growth

in rats. The rats were made hyperphagic by food deprivation for 4 h at the beginning of the dark cycle.

Mennini et al. (1991) and Anelli et al. (1992) studied the anorectic activity of various compounds in different species, such as mice, rats, and guinea pigs.

Bowden et al. (1988) used metabolism cages equipped with automated feeding monitors. Food was provided as 45 mg pellets which were singly delivered to a feeding trough. A photodetector sensed the removal of the pellet, and the number of pellets delivered over a specified time interval was recorded.

Samanin et al. (1979) described anorexia in rats induced by the central serotonin agonist m-chlorophenylpiperazine.

Blavet et al. (1982) studied food intake in fasted rats after treatment with several typical anorexigenic agents.

Dourish et al. (1985) investigated the effects of the serotonin agonist 8-OH-DPAT on food intake in non-deprived male rats. This effect was prevented by p-chlorophenylalanine (Dourish et al. 1986).

The anxiolytics gespiron, buspirone and ipsapirone increased free feeding in rats and did not inhibit feeding induced by 8-OH-DPAT (Gilbert and Dourish 1987).

Jackson et al. (1997) investigated the mechanisms underlying the hypophagic effects of the 5-HT and noradrenaline reuptake inhibitor, sibutramine, in the rat.

Simansky and Vaidya (1990) tested the anorectic action of a serotonin uptake inhibitor by measuring the volume of milk consumed by food-deprived rats.

Stevens and Edwards (1996) induced anorexia by subcutaneous injection of 5 mg/kg 5-hydroxytryptamine in Wistar rats habituated to a restricted feeding regime and tested the effects of a 5-HT₃ antagonist.

Rouru et al. (1992) investigated in genetically obese male Zucker rats the effect of subchronic metformin treatment on food intake, weight gain and plasma insulin and corticosterone levels and somatostatin concentrations in the pancreas.

Robert et al. (1989) found an enhanced food intake after intracerebroventricular administration of the tetrapeptide FMRF-amide (Phe-Met-Arg-Phe-NH₂) in obese “cafeteria” rats.

Cooper et al. (1990a, b) used non-deprived rats to study anorectic effects in a test of palatable food consumption and in nocturnal free-feeding.

Cooper et al. (1990c) tested not only food consumption but also the frequency of feeding bouts and duration of individual feeding episodes.

Eberle-Wang and Simansky (1992) studied the influence on the anorectic action of CCK and serotonin by measuring the uptake of sweetened mash mixture in rats.

Voigt et al. (1995) studied the involvement of the 5-HT_{1A} receptor in CCK induced satiety by recording food intake during a 2 h test meal in food deprived and in freely feeding rats.

Influence on postprandial satiety in rats was tested by Rosofsky and Geary (1989). Rats were given pelleted chow and water ad libitum. Near the middle of the bright phase of the light-dark cycle, pellets were removed, the animals treated and condensed milk presented 30 min later. Milk consumption was measured at 4-min intervals for 40 min.

Rats show a dramatic and reliable reduction of food intake if they are prefed a low-protein basal diet and then offered a diet that is imbalanced in any of the essential amino acids (Leung and Rogers 1969). This anorectic response has been used by Hammer et al. (1990) to test serotonin₃ receptor antagonists.

Tail pinch is an effective stimulant of eating in rats (Antelman and Szechtman 1975; Fray et al. 1982). Clark et al. (1992) reported that N-methyl-D-aspartate lesions of the lateral hypothalamus do not reduce amphetamine or fenfluramine anorexia but enhance the acquisition of eating in response to tail pinch in the rat.

Thurlby and Samanin (1981) studied the effect of anorectic drugs on food-rewarded runway behavior.

Ferrari et al. (1992) studied the effects on anorexia induced by ACTH and immobilization in rats in an X-maze with alternate open and covered arms, each baited with laboratory chow.

Cooper et al. (1993) studied dopamine D-1 receptor antagonists in rats with chronic gastric fistula which were trained to sham-feed a 10% sucrose solution in a 60 min test.

Bickel et al. (2004) analyzed anorectic effects in rodents by measurement of food consumption. Mice or rats were individually placed in Macrolon cages equipped with a device for continuous monitoring of food consumption consisting of a container filled with feed, hanging on an electronic balance. The balance transmits the weight changes of the feed container continuously to a central unit. Data were stored and processed at the end of the experiment. This system, obtained from TSE, Bad Homburg, Germany, can measure food consumption for up to 3 weeks from more than 199 feeding sensors simultaneously. Similar equipment was used by Bauhofer et al. (2002) and Gaetani et al. (2003).

In wild rodents, **hoarding of food** covers the long term alimentary need. In the laboratory, hoarding behavior does not occur in ad libitum fed rats. On the contrary, rats whose energy balance is threatened by previous food restriction hoard as soon as experimental conditions allow to do so. When such a rat gets free access to a food stock (placed outside its usual territory), it carries food into its shelter and accumulates an amount proportionate to its body weight. Fantino et al. (1980, 1986, 1988), Nishida et al. (1990) used the reduction of the amount of food hoarded during a period of 3 h as parameter for anorectic activity of drugs.

Caccia et al. (1993) studied the anorectic effect of D-fenfluramine in the **marmoset** (*Callithrix jacchus*).

Knoll (1979, 1984) described satietin, a anorectic substance which has been isolated from serum of humans and several animal species. Satietin has been reported to be a 50000–70000 dalton MW glycoprotein, containing 14–15% amino acids and 70–75% carbohydrates, surviving digestion with proteases and boiling. Purification of bovine serum satietin was reported by Nagy (1994). These factors suppressed feeding in rats after peripheral and after intracerebroventricular administration.

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L.3.1.2**Computer-Assisted Measurement of Food Consumption in Rats****PURPOSE AND RATIONALE**

Techniques have been developed for continuous measurement of food consumption in rats.

PROCEDURE

Animals are placed individually in cages (Macrolon-cage Type 4, size: 44 × 26 × 15 cm) equipped with a device for continuous monitoring of feed consumption. This device is a container filled with feed and hanging on an electronic balance in the cage. The balance transmits the weight changes of the feed container continuously to a central unit. The data are stored and processed at the end of the experiment. This system can measure feed consumption continuously for up to 2 weeks from up to 64 feeding sensors (animals) simultaneously. The feeding monitoring system and data processing hardware and software (Release V3.07) can be obtained from TSE-Systems, Bad Homburg, Germany. Software options: the data are presented in the form of a matrix. The matrix delivers cumulatively or sequentially feed consumption in grams (g) for given time intervals for each individual animal. The advantages of this system are that the animals are kept in their home cages, that the feed monitoring period is long and, most of all, the animals are left undisturbed during the experimental session. An additional program for microstructural analysis calculates the parameters for feed consumption over a given time (24 h). Parameters delivered are: number of meals (Nm/time), inter-meal breaks (IMB) (min), average meal size (g) and average meal duration (min). In the present study, a meal was defined to be ≥ 0.5 g and the IMB ≥ 15 min. Using this system we have determined the anorectic activity of the central acting anorectic agents amphetamine, sibutramine, and dexfenfluramine (Bickel et al. 2000), as well as the peripheral acting anorectic SR146131, a CCK_A-receptor antagonist (Bickel et al. 2002), and HMR1426, an anorectic drug that powerfully inhibits gastric emptying rate (Bickel et al. 2004).

A 2-week treatment with HMR1426 in Zucker fatty rats fed a normal standard rat diet (1 g feed = 12.1 kJ) reduced the body weight increase significantly by 5% (Gossel et al. 2001). In Zucker fatty rats fed a fat-rich diet (1 g feed = 20.2 kJ), 3 weeks of treatment with HMR1426 caused a significant loss of body weight of 33% (Bickel et al. 2003).

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L.3.1.3**The Endocannabinoid System****PURPOSE AND RATIONALE**

Recent discovery of cannabinoid receptors and their natural ligands, the endocannabinoids, has initiated the development of synthetic antagonists and agonist of these receptors to better understand the physiological role of the endocannabinoid system. Two types of receptors have been so far identified: the CB₁-receptor present in brain and periphery and the CB₂-receptor found in the periphery only. Both types of receptors are negatively coupled to Ca²⁺ channels through G-proteins (Pertwee 1997; Howlett et al. 2002). Kogan and Mechoulam (2006) gave an overview on the chemistry of endocannabinoids as well as of some synthetic molecules that affect the endocannabinoid system. Matias and Di Marzo (2006) described the enzymes involved in endocannabinoid synthesis and degradation. The endocannabinoid system has emerged as an important neuromodulatory system in the brain (Marsicano and Lutz 2006). Endocannabinoids play important roles in the gastrointestinal tract (Massa and Monory 2006) and in energy metabolism (Pagotto and Pasquali 2006).

For further studies on the pharmacological effects of cannabinoids and cannabinoid antagonists and on cannabinoid receptor binding see Sects. H.1.1.8, H.1.1.8.1, and H.1.1.8.2.

The appetite-stimulating effect of marijuana with its main active ingredient Δ^9 -tetrahydrocannabinoid (Δ^9 -

THC) has been known for a long time. The demonstration that the endogenous endocannabinoid anandamide induces overeating in rats suggests a physiological role for endocannabinoids (Williams and Kirkham 1999). Further support for this hypothesis came from CB1 receptor knockout mice (CB1^{-/-}) which were hypophagic and lean compared to wild-type mice (Cota et al. 2003).

The search for CB₁-receptor antagonists with anorectic properties led to the discovery of SR141716 (rimonabant). The compound inhibited CB₁-receptors in rat brain synaptosomes with an IC₅₀ = 2.35 ± 0.47 nM (Rinaldi-Carmona et al. 1996). A large number of different investigators have confirmed the powerful anorectic and antiobesic effect of this drug in various models. Simiand et al. (1998) investigated the anorectic effect in marmosets. Colombo et al. (1998) tested the anorectic effect and body weight reduction in non-obese Wistar rats. Ravinet-Trillou et al. (2003) showed anorectic properties of SR141716 in diet-induced obese mice.

PROCEDURE

Mice were housed on a reverse light-dark cycle (lights off 0900–2100 hours) in a room with temperature (22 ± 2°C) and humidity control. They were fed either a high-fat diet (HFD) of 4.7 cal/g energy density (TD 97366, Harlan; 49% fat, 18% protein, 33% carbohydrate) or a standard mouse diet (STD) containing 2.9 kcal/g (A04C, UAR; 8% fat, 19% protein, 73% carbohydrate). SR141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide] was administered orally in distilled water with 0.1% Tween 80 just before the onset of the dark phase. A habituation to treatment with the vehicle was performed during the week before the start of treatment.

SR141716 Treatment in DIO Mice

Six-week-old C57BL/6J male mice were given HFD or STD diets for 12–17 weeks before drug treatment started. The mice were weight matched and assigned to one of the following three groups: high-fat diet fed/vehicle-treated (HFD-Veh), high-fat diet fed/SR141716-treated (10 mg/kg) (HFD-SR 10 mg), and standard-diet fed/vehicle-treated (STD-Veh). Body weight and individual food intake were recorded daily. The energy intake was determined taking into account the caloric density of each diet and the average daily energy intake was calculated weekly for each of the 5 weeks of treatment. To balance the wide difference in body weight between the groups,

the relative energy intake was calculated by correcting for body weight and was expressed as kilocalories per gram mouse per day × 100. After a 30-day treatment, body fat and lean masses of anesthetized mice were estimated using a small research animal body composition analyzer (model SA-3000, EM-SCAN). The measurement principle is based on the high electrical conductivity in all lean tissues relative to lipids when exposed to an oscillating radio frequency. We previously calibrated the device by using carcass analysis of mice fed a standard or a high-fat diet. Regression models integrating total body electrical conductivity (TOBEC) values and body weights were fitted to chemically analyzed whole-body protein and fat contents (adjusted $r^2 = 0.96$, $P < 0.0001$ for protein, adjusted $r^2 = 0.93$, $P < 0.0001$ for fat). Food deprivation was shown to produce a reduction of metabolic rate in rodents and body weight loss during fasting was reported to be an indicator of this metabolic rate. Thus body weight change during a 24-h fast was measured after 5 weeks of treatment. Finally, additional 18-h-fasted mice were injected intraperitoneally with human recombinant insulin (Sigma) at the dose of 0.6 U/kg. Glycemia was measured before and 30, 60, 90, 120, and 180 min after insulin injection using a glucose analyzer (Bayer Diagnostics) to assess insulin sensitivity.

Another experiment compared a 3-day SR141716 treatment with a caloric restriction. Obese mice were randomized into one of the three treatment groups: a group treated with vehicle, a group treated with SR141716 at the dose of 10 mg/kg per day for 3 days, and a vehicle-treated pair-fed group (HFD-PFVeh) that was fed the same quantity of diet as that consumed by the animals receiving SR141716. Food intake and body weight were monitored daily; lumbar white adipose pads were weighed after death.

SR141716 Treatment in CB1 Receptor Knockout Mice

CB1 receptor knockout (CB1^{-/-}) mice were generated. For constructing the targeting vector, a 9-kb *KpnI-KpnI* fragment including the entire coding region of the CB1-receptor gene was cloned from a 129SvJ6 mouse genomic library. An *ApaI-ApaI* fragment containing part of the coding region was inactivated by substitution with a neomycin resistance cassette. The targeting vector was transfected into embryonic stem cells from the 129/Ola line. Then, mutated ES cells were injected into C57BL/6 blastocytes to generate chimeric founder mice. Germline transmission of the targeted allele was determined by PCR analysis of mouse tail genomic DNA. The homozygous CB1^{-/-} mice used in this work were on a C57BL/6X129/Ola

F2 genetic background. The lack of binding of the synthetic CB1 receptor agonist [^3H]CP55,940 in these mice was checked by autoradiography in various brain sections. After 6 weeks of a high-fat diet, 15-week-old male $\text{CB1}^{-/-}$ mice were either given SR141716 orally at the dose of 10 mg/kg per day or treated with vehicle alone. Body weight and food intake were monitored daily throughout the 3-week treatment period as previously described.

EVALUATION

ANOVA and post hoc analyses were performed using SAS version 8.2. A repeated two-way ANOVA for treatment and time (repeated measures on food intake and body weight) or one-way ANOVA for treatment (all other variables) was used to determine statistical significance. When a single dose of SR141716 was included in the experiment, post hoc comparisons between the SR141716-treated group, obese, and lean control groups were performed after Holm's adjustment. When two doses of SR141716 were studied, comparisons among dietary obese mice receiving each of SR141716 doses versus vehicle were performed by Dunnett's test. When the hypotheses necessary for the application of parametric tests were not achieved, appropriate transformations were applied. The results are presented as means \pm SEM.

MODIFICATIONS OF THE METHOD

SR147778, a CB1 -receptor antagonist, was shown to cause a long-lasting anorectic effect in fasted and non-deprived rats and to reduce ethanol drinking in mice. In CHO-cells expressing the $h\text{CB1}$ -receptor the K_i was 3.5 ± 0.29 nM, whereas the K_i for $h\text{CB2}$ -receptors was 442 ± 30 nM (Rinaldi-Carmona et al. 2004).

Animals

Male Sprague-Dawley rats (180–250 g) were used for *in vitro* and food intake studies. Male Wistar rats (300 g) obtained from Charles River were used for schedule-induced polydipsia (SIP) studies.

Drug Preparation and Administration

For *in vivo* experiments, drugs were suspended with 0.01% Tween 80 plus DMSO in saline (NaCl 0.9%) for i.v. administration or distilled water for other routes. SR147778 was administered by i.p., p.o., or s.c. routes in a volume of 20 ml/kg body weight.

Voluntary Ethanol Consumption

The test was performed as described by Arnone et al. (1997). One week before testing, mice were singly

housed in cages equipped with two bottles of plain water. Mice were then subjected to five daily 6-h test sessions during which they had a free choice between one bottle of water or one filled with a 10% (v/v) non-sweetened ethanol solution. The quantities of the different liquids consumed were measured by weighing the bottle before and after each session. The bottles were regularly checked for possible leakage. SR147778 was administered 30 min before each experiment session.

Spontaneous Sucrose Drinking

Two weeks prior to testing, rats caged individually were given access to a sucrose solution (5%) in their home cage during daily 4-h sessions (without food and water). At the end of the training periods, rats displaying a poor sucrose drinking response were discarded from the study. On the day of the test, rats were administered orally with SR147778 or vehicle in a volume of 2 ml/kg 1 h before the presentation of the sucrose solution. The amount of the sucrose solution drunk by each rat ($n = 6-8$ per group) was measured by weighing each bottle before and at the end of the 4-h drinking period.

Food Intake in 18-h Fasted Rats

Over a period of 10 days before the experiment, rats were fasted for 18 h and allowed access to food for only 6 h between 10:00 a.m. and 4:00 p.m. each day. Water was available ad libitum. At the end of this adaptation phase, SR147778 or vehicle was administered orally, in a volume of 5 ml/kg, at 9:00 a.m., 1 h after a preweighed amount of food (A04 standard rat laboratory diet) was introduced into the cage, and then food intake was measured (taking into account spillage) at different times.

Spontaneous Feeding in Non-deprived Rats

Rats were maintained in a reversed light/dark cycle (lights off 9:00 a.m. to 9:00 p.m.) with food and water ad libitum. On the day of the experiment, food was removed from the cage at 8:00 a.m., and animals were administered orally with SR147778 or vehicle in a volume of 5 ml/kg at 9:00 a.m. (at the beginning of the dark phase). One hour after, a preweighed amount of food (A04 standard rat laboratory diet) was introduced into the cage, and then food intake was measured (taking into account spillage) at different times.

Schedule-Induced Ethanol Polydipsia

Rats were randomly allocated to drinking either water or ethanol in a protocol of SIP (Falk et al. 1972). They were food deprived (food available for 1 h per

day) but not water deprived. They were trained in operant chambers (40.5 × 31 × 31 cm; Imetronic, Pessac, France) fitted with a food tray where food pellets (45 mg; Bioserv, Phymep, Paris) were delivered every 60 s (FT-60s). Visits to the food tray were automatically recorded. A metal drinking tube protruded into the chamber near the food magazine and was connected to a drinking bottle. Licks of the drinking tube were numbered by the closure of an electric circuit. Bottles were weighed to the nearest 0.1 g at the end of each SIP session. Both groups were trained for 2 weeks, 5 days per week, under the SIP procedure until they drank about 10 ml of plain water, which was then substituted for slightly sweetened solutions (0.05% weight/volume saccharose) of either water or ethanol. Over the next 2 weeks of training, the concentration of ethanol was progressively increased by steps of 2% to reach the final 10% concentration. Fluid consumption was allowed to stabilize over five daily training sessions. Then, each group (ethanol or water) was divided into two subgroups: one treated with one dose of SR14778 administered i.p. 1 h before testing and the other subgroup administered with the corresponding vehicle. As a control, SR141716 was tested at 10 mg/kg in the same conditions. The four subgroups of rats were tested once a week (1-h test session) with a different dose of SR14778 and trained daily (1-h training session) on the 4 remaining days. In the same rats and in the same experimental conditions, the activity of the reference CB1 antagonist, SR141716, had previously been investigated at 10 mg/kg i.p. Data are presented as the mean quantities of water or ethanol consumed during each test session; they were analyzed with Student's *t*-test to compare the quantities of water or ethanol rats drank after administration of SR14778 or vehicle during a given test session.

Data Analysis

Data from competition experiments (IC_{50} , EC_{50} , and DE_{50}) were analyzed using a non-linear least-squares method. K_d values of 0.09 ± 0.01 nM (brain), 0.309 ± 0.031 nM (hCB1), 0.17 ± 0.03 nM (spleen), and 0.49 ± 0.11 nM (hCB2) were used to determine K_i values. A Schild plot was constructed to estimate the pA_2 value in mouse vas deferens and adenylyl cyclase studies. Statistical significance was determined by Student's *t*-test, and $p < 0.05$ was considered significant. For *in vivo* pharmacological tests, statistical analysis was performed using ANOVA followed by Dunnett's *t*-test. ID_{50} values were calculated using the Pharmacofit method.

Matias et al. (2006a) described regulation of the levels of endogenous cannabinoids in the brain and peripheral tissues and the control of food intake.

Mechoulam et al. (2006) reviewed the effects of endocannabinoids on feeding and suckling.

Regulation, function and dysregulation of endocannabinoids in models of adipose and β -pancreatic cells and in obesity and hyperglycemia are described by Matias et al. (2006b).

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L.3.2

Metabolic Activity

L.3.2.1

GDP-Binding in Brown Adipose Tissue

PURPOSE AND RATIONALE

Brown adipose tissue is the major site for non-shivering thermogenesis in rodents (Ricquier and Mory 1984; Foster 1986). Drugs activating brown adipose tissue thermogenesis via β -adrenoceptors cause uncoupling of oxidative phosphorylation from electron transport (Arch et al. 1984; Nicholls et al. 1986). The binding of the nucleotide guanosine diphosphate (GDP) to brown adipose tissue membrane protein, the uncoupling protein or thermogenin (Ricquier and Bouillaud 1986), is an established indicator of the thermogenetic activity of brown adipose tissue (Milner et al. 1988).

PROCEDURE

Obese male fatty Zucker rats at the age of 13 weeks weighing about 450 g receive various doses of the test compound in the drinking water or tap water as control for 21 days. Food intake is measured every day and body weight every other day. At the end of the treatment, the rats are sacrificed by decapitation and interscapular brown adipose tissue is quickly dissected from surrounding tissue.

According to the method published by Nicholls (1976) brown adipose tissue is minced, diluted with 250 mM sucrose and homogenized with a Potter S homogenizer (B. Braun Melsungen, Germany). The homogenate is centrifuged for 10 min at 8500 g. The pellet is diluted with 250 mM sucrose and centrifuged at

700 g for 10 min. The supernatant is collected and centrifuged at 8500 g for 10 min. Bovine serum albumin at 0.2% is added to wash the suspension of the pellet, which now consists of mitochondria. After centrifugation (8500 g) the resulting pellet is suspended in albumin-free sucrose buffer. The binding of [3H]-GDP (DuPont NEN, Boston MA) to mitochondria of single rats is determined by incubating mitochondria in a basic medium containing 100 mM sucrose, 20 mM TES (*N*-tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid), 1 mM EDTA, 10 mM choline chloride, 2 μ M rotenone, [¹⁴C]sucrose (0.125 μ Ci ml⁻¹) and 10 μ M [3H]-GDP (0.53 Ci mmol⁻¹) at room temperature. Non-specific binding is assessed in the presence of unlabelled GDP (1 mM). After 10 min of incubation, the reaction is terminated by filtration the mixture through Thomas A/E glass fibre filters by using a Brandel cell harvester. The radioactivity is measured with the Opti Phase 'High Safe' II scintillation cocktail and a scintillation counter enabling the samples to be counted for both ³H and ¹⁴C. The protein content of the mitochondrial suspensions is assayed according to the method of Peterson (1977).

EVALUATION

GDP binding is assessed from ³H radioactivity with a correction for trapped medium using [¹⁴C]-sucrose. Two way analysis of variance (ANOVA) is performed.

MODIFICATIONS OF THE METHOD

Glucose is a minor substrate for isolated brown adipocytes, fuelling thermogenesis by a maximum of 16% (Isler et al. 1987).

The mechanism of anti-obesity action of a dihydropyridine calcium antagonist was studied by Yoshida et al. (1994b). Obesity was induced by subcutaneous injection of 2 g/kg monosodium-L-glutamate immediately after birth for 5 consecutive days in ICR female mice. Binding of GDP and cytochrome c oxidase activity in brown adipose tissue mitochondria were significantly increased after 4 weeks drug treatment incorporated in the diet.

Yoshida et al. (1984, 1985) found a reduced norepinephrine turnover in brown adipose tissue of mice with monosodium glutamate-induced obesity.

Kajita et al. (1994), Takahashi et al. (1994) determined regional blood flow in brown adipose tissue by the microsphere method in anesthetized rats.

Yoshida et al. (1996) determined the effect of mazindol on noradrenaline turnover in brown adipose tissue of yellow KK mice and C57B1 control mice.

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L.3.2.2**Uncoupling Protein and GLUT4 in Brown Adipose Tissue****PURPOSE AND RATIONALE**

Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane transporters which dissipate the proton gradient, releasing stored energy as heat. UCP1 is expressed exclusively in brown adipocytes, UCP2 is expressed widely, while UCP3 is found in skeletal muscle and brown adipose tissue (Vidal-Puig et al. 1997; Fleury et al. 1997; Masaki et al. 1997; Matsuda et al. 1997; Boss et al. 1997; Gong et al. 1997; Larkin et al. 1997). They are upregulated by thyroid hormones (Larkin 1997; Masaki 1997; Branco et al. 1999; Lanni et al. 1999). Mao et al. (1999) identified and characterized a novel member of the human uncoupling protein family, termed uncoupling protein-4 (UCP4).

In addition to the binding of the nucleotide guanosine diphosphate (GDP) to brown adipose tissue membrane protein, the uncoupling protein itself, and the glucose transporter 4 (GLUT4) were determined by RNA (Northern blot) analysis and by protein (Western blot) analysis as indicators of the thermogenetic activity of brown adipose tissue.

PROCEDURE

Male fatty (OLETF fatty) rats at the age of 10 weeks are given subcutaneous injection of test compound or

solvent once daily. After 14 weeks treatment, the rats are sacrificed and brown and white adipose tissue samples rapidly removed.

Northern Blot Analysis

Total RNA is extracted from 0.1–1 g of tissue using TRIzol (Gibco) and the concentration determined from the absorbance at 260 nm. Total RNA (20 mg) is separated on a 1.5% agarose/formaldehyde gel, and transferred to and fixed on a nylon membrane. A 488 bp uncoupling protein cDNA probe corresponding to the coding region of rat uncoupling protein is prepared by digesting the whole uncoupling protein cDNA with BamHI. The uncoupling protein probe and GLUT4 cDNA are labeled with α -[³²P]dCTP (deoxy-cytidine-triphosphate). The blots are hybridized to the labeled probes at 42°C for 20 h in the presence of 500 µg/ml salmon sperm DNA, and exposed to an X-ray film for autoradiography and an imaging plate of BAS1000 (Fuji Film) for quantitative analysis.

Western Blot Analysis

Each tissue is homogenized in 5–10 volumes of a solution containing 10 mmol/l Tris-HCl and 1 mmol/l EDTA (pH 7.4) for 30 s with a Polytron. After centrifugation at 1500 g for 5 min, the fat cake is discarded, and the infranant (fat-free extract) is used for protein determination according to Lowry et al. (1951) and cytochrome C oxidase activity (Yonetani and Ray 1965). Uncoupling protein and GLUT4 protein in the fat-free extract is measured by Western blot analysis. The fat-free extracts (10 µg protein of brown adipose tissue, 20 µg of white adipose tissue) are solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose filter. After blocking with 5% non-fat dry milk, the filter is incubated with rabbit antiserum against rat uncoupling protein or GLUT4. The rabbit antisera against rat uncoupling protein and GLUT4 are prepared by immunizing purified rat uncoupling protein (Lin and Klingenberg 1980) and a 12-amino acid peptide corresponding to GLUT4 (residues 498–509, TELEYL-GPDEND) (Shimizu et al. 1993) respectively, coupled with keyhole limpet hemocyanin. Then the filter is incubated with [¹²⁵I]protein A (ICN). The dry blot is exposed to an X-ray film for autoradiography and an imaging plate of BAS1000 (Fuji Film) for quantitative analysis.

EVALUATION

Data are expressed as means \pm SEM and are analyzed by two-way ANOVA followed by Bonferroni *t*-test.

MODIFICATIONS OF THE METHOD

A radioimmunoassay to measure uncoupling protein was used by Milner et al. (1988).

Scarpace et al. (2000) performed unilateral surgical denervation of the interscapular BAT in rats under pentobarbital anesthesia according to Bartness et al. (1986). A transverse incision was made just anterior to the BAT, separating the BAT from the muscles of the scapulae. The BAT was raised to expose the five intercostal nerve bundles entering each pad. On one side, a section of each nerve bundle was removed with scissors. Denervation can be verified by assessing norepinephrine levels in the innervated compared with the denervated BAT pads (Scarpace and Matheny 1996).

Puigserver et al. (1996) studied the effects of retinoic acid isomers on the appearance of uncoupling protein in primary cultures of brown adipocytes, in the brown adipocyte cell line HIB 1B and directly in intact mice.

Shimabukuro et al. (1997) reported the induction of uncoupling protein-2 mRNA in the pancreatic islets of Zucker diabetic fatty rats by troglitazone.

Foellmi-Adams et al. (1996) found a synergy between norepinephrine and pioglitazone in induction of uncoupling protein in mice.

Kotz et al. (2000) determined uncoupling protein 1 (UCP1) in brown adipose tissue, UCP2 in white adipose tissue and UCP3 in muscle of male Sprague Dawley rats after injection of neuropeptide Y into the hypothalamic paraventricular nucleus.

Great efforts have been devoted to study the influence of **β_3 -adrenergic agonists** on formation of uncoupling protein.

Umekawa et al. (1997) found an induction of uncoupling protein and activation of GLUT4 in white fat of Otsuka Long-Evans Tokushima fatty rats after treatment with a specific β_3 -adrenoceptor agonist.

Ghorbani and Himms-Hagen (1997) found appearance of abundant densely-stained brown adipocytes expressing uncoupling protein in white adipose tissue during reversal of obesity and diabetes in Zucker fa/fa rats induced by the β_3 -adrenoceptor agonist CL316,243.

Nagase et al. (1996) found in yellow obese KK mice after treatment with a β_3 -adrenergic agonist a significant reduction of body weight, associated with a marked decrease of white fat pad weight and hypertrophy of the interscapular brown adipose tissue with a sixfold increase in mitochondrial uncoupling protein content.

Liu et al. (1995, 1996), Stock (1997) reported an increase of glucose utilization in rat brown adipose tis-

sue after treatment with a β_3 -adrenoreceptor agonist or with sibutramine, a serotonin and noradrenaline reuptake inhibitor.

Savontaus et al. (1998) reported an increase of UCP3 and UCP1 in brown adipose tissue of obese fa/fa Zucker rats after chronic administration of a β_3 -adrenoreceptor agonist.

The effects of β_3 -adrenoceptor agonists on uncoupling protein-1 and leptin in culture-differentiated rat brown fat cells are antagonized by a β_3 -adrenoreceptor antagonist (Tonello et al. 1998).

Berraondo et al. (2000) found an up-regulation of muscle UCP2 gene expression by a β_3 -adrenoreceptor agonist in obese rodents, but down-regulation in lean animals.

Paulik and Lennard (1997) found an increased expression of uncoupling protein in C3H10T1/2 cells, a pluripotent stem-cell line, after addition of **thiazolidinediones**.

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L.3.2.3

Resting Metabolic Rate

PURPOSE AND RATIONALE

Resting metabolic rate can be influenced by various drugs both in normal and obese animals.

PROCEDURE

Female yellow KK mice and female C57B1 mice at the age of 12 weeks are housed under controlled temperature and artificial light/dark conditions and are fed with commercial powdered chow and tap water ad libitum. Groups of each strain are treated with doses of test drug or solvent by daily intramuscular injections for 2 weeks. Daily food intake and body weight is measured.

Resting metabolic rate is estimated by means of a closed-circuit metabolic system (Molnar et al. 1986). The system consists of a chamber, circulating pump, desiccant and CO₂-absorbent canisters, solenoid valve, regulated gas source, and modified liquid crystal display calculator. The internal volume of a chamber for a single rat should be 4.5 liters. Food cups are placed on the door for convenient access when refilling. Wa-

ter is supplied in a tray filled intermittently by means of a stopcock in the chamber wall. A shallow stainless steel funnel-shaped tray for urine collection is located in the bottom of the chamber.

Animals are placed in the chamber and the door is sealed airtight. Chamber air circulates by means of a peristaltic pump at a rate of 2.3 l/min through an external loop containing canisters of desiccant and CO₂-absorbent. In this manner, water vapor pressure remains constant and the respiration by-product, CO₂, is removed. Consumption of O₂ produces a pressure drop in the sealed system which actuates the pressure sensor for O₂ replacement.

The pressure sensor has two input ports, low and high, producing a simple contact closure whenever the high pressure exceeds the low by ~1 Torr. The low port is connected to the interior of the metabolic chamber by a length of tubing attached to a threaded connector, whereas the high port is open to the atmosphere. Thus the contact closes whenever the pressure within the chamber falls to more than 1 Torr below atmospheric pressure.

The contact closure is routed to the control unit where a precise pulse of fixed duration is triggered. This pulse activates a solid-state relay through an emitter-follower transistor circuit. The relay output is in series with a solenoid valve and line voltage, so that contact closure results in opening of the valve for a fixed duration. Since the input port of the valve is at a constant pressure, a predictable, highly repeatable volume of gas flows into the chamber. This raises the chamber pressure and opens the pressure sensor contacts. The entire cycle repeats after sufficient metabolism to again decrease the chamber pressure and close the sensor contacts.

After a stable baseline has been achieved after 30 min, drugs are administered and resting metabolic rate is measured for one hour at an ambient temperature of 22°C.

EVALUATION

Data are presented as means \pm SEM. Statistical analysis is performed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Poon and Cameron (1978) measured oxygen consumption utilizing a standard Machlett manometer connected to a desiccator that accommodated a single mouse. The desiccator was placed in a 20°C water bath to ensure constant temperature throughout and CO₂ was absorbed by means of soda lime UPS. After a 5-min equilibration period, measurements of oxy-

gen consumption were obtained over a 5-min period. The procedure was repeated twice within 1 h for each mouse.

In order to study differential antagonism to amphetamine-induced oxygen consumption and agitation by psychoactive drugs Niemegeers and Janssen (1979) placed groups of 3 rats with a total weight of 235 ± 5 g consisting of 2 parts, a bottom and a cover, both made up from clear glass. The cover, containing a thermometer, was used to seal the chamber airtight. Inside the chamber a perforate plate was placed as animal platform. Containers filled with calcium oxide were placed below and above the animal holder. The chamber was flushed with oxygen for 3 min at an overpressure of 50 units as measured by a manometer connected to one outlet of the chamber. Test drugs were administered subcutaneously 60 min before the animals were placed in the test chambers and 45 min before 2.5 mg/kg amphetamine were injected. Oxygen consumption was measured as decrease of manometer pressure over one hour.

Rothwell (1989) measured central activation of thermogenesis by prostaglandins by resting oxygen consumption in individual closed-circuit calorimeters (Stock 1975) and by registration of colonic temperature in conscious rats.

Jensen et al. (2001) described a self-correcting indirect calorimeter system for the measurement of energy balance in small animals.

Himms-Hagen et al. (1994) tested the effect of a thermogenic β_3 -agonist on energy balance in rats. Twenty-four hours energy expenditure, resting metabolic rate, and thermic effect of food were measured using open-circuit indirect calorimetry. The rat was placed in a respiration chamber ($44 \times 22 \times 18$ cm), airflow was measured with a Brooks thermal mass flowmeter (Brooks Instrument Division, Emerson Electric, Hatfield, PA), and the flow of outside air through the chamber was controlled at a variable rate by two adjustable peristaltic pumps that maintained CO_2 concentration at $<1\%$. Temperature was maintained at $23 \pm 1.0^\circ\text{C}$. Humidity was measured by an electronic psychrometer, barometric pressure was recorded, and feeding activity was monitored by an infrared detector. The rat was placed into the chamber at 09:30 and removed at 08:30 at the following morning. Food was available to the rat only between 16:30 and 07:30. Total energy expenditure was measured for 23. Resting metabolic rate was determined from the lowest energy expenditure at rest between 11:00 and 16:00. Thermic effect of food was determined from the difference between resting metabolic

rate and the lowest energy expenditure at rest between 24:00 and 07:00. For the measurement of minimum metabolic rate, the rat was anesthetized (pentobarbital sodium, 60 mg/kg) and then placed in a small chamber filled with warm circulating water in which it was submerged except for the head. Core temperature was measured with a digital thermometer immediately after induction of anesthesia, and this temperature was maintained ($\pm 0.1^\circ\text{C}$) by adjusting the temperature of the circulating water bath. The chamber was sealed and minimum metabolic rate determined with the same equipment as was used for the 24-h energy expenditure. Measurements of minimum metabolic rate were made for 5–15 min between 08:30 and 09:30 after a stabilization period of 5–10 min.

Ghorbani et al. (1997) tested the effect of a β_3 -adrenoreceptor agonist on resting metabolic rate in rats by placing the animal in a water-jacketed chamber at 28°C , volume 1 liter, through which air, also at 28°C , was drawn at a rate of 1 l/min. Oxygen entering and leaving the chamber was measured with an oxygen analyzer (Beckman Industrial Oxygen Analyzer, model 755).

Paulik et al. (1998) described a robust technique to measure thermogenesis of **yeast cells** cultured in microtiter plates using infrared thermography. Thermogenesis increased after exposing yeast to uncoupling protein-2, or troglitazone or β_3 -adrenoreceptor agonists.

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L.3.2.4

β_3 -Adrenoceptor

PURPOSE AND RATIONALE

β_3 -adrenoreceptor agonists produce weight loss in obese rodents (Yoshida et al. 1994; Weyer et al. 1999) which is almost entirely accounted for a reduction in body fat. As this effect is observed without a decrease in food intake, it is thought to be due to increased thermogenesis in brown adipose tissue, increased lipolysis in white adipose tissue, and suppression of leptin gene expression and serum leptin levels (Arch and Wilson 1996; Kumar et al. 1999).

The β_3 -adrenoreceptor has been cloned and characterized in animals (Granneman et al. 1991; Nahmias et al. 1991; Evans et al. 1996) as well as from man (Emorine et al. 1989; Strosberg 1997). However, the physiological function of the human β_3 -adrenoreceptor, the significance of animal data with β_3 -adrenoreceptor agonists, and even the presence of a putative fourth β -adrenoreceptor in human adipose tissue are still a matter of debate (Emorine et al. 1994; Galitzky et al. 1998; Kaumann et al. 1998; Sarsero et al. 1998; Strosberg et al. 1998; Weyer et al. 1999).

The development of a Chinese hamster ovary cell transfection system, using the human β_3 -adrenoreceptor gene resulted in potential new selective β_3 -agonists being identified (Carruba et al. 1998). Human β_1 -, β_2 - and β_3 -adrenoreceptors expressed in Chinese hamster ovary (CHO) cells were used to evaluate potential β_3 -adrenoreceptor agonists by He et al. (2000).

PROCEDURE

cAMP Response Element-Luciferase Receptor Gene Assay

CHO cells stably expressing human β_1 -, β_2 - or β_3 -adrenoreceptor populations are transfected with cAMP response element-luciferase plasmids using electroporation with a single 70-ms, 150-V pulse (Vansal and Fellner 1999). The transfected cells are seeded at

a density of 40000/well in 96-well microtiter plates and allowed to grow for 20 h. After 20 h, the cells are treated with varying drug concentrations (10^{-11} to 10^{-4} M) for 4 h. Following drug exposure, the cells are lysed and luciferase activity is measured using the Lu-cLite assay kit (Packard). Changes in light production are measured by a Topcount luminometer (Packard).

EVALUATION

Data are analyzed in duplicate at each concentration and expressed as percent luciferase response relative to the maximum response to (–)-isoproterenol (10^{-6} M). Results are expressed as the mean \pm SEM.

MODIFICATIONS OF THE METHOD

Gettys et al. (1996) reported that the β_3 -adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat white adipocytes.

Using reverse transcription-polymerase chain reaction, Evans et al. (1998) compared levels of β_3 -adrenoreceptor mRNA in white adipose tissue, brown adipose tissue, ileum and colon from genetically obese (*ob/ob*) and lean (+/+) C57BL/6J mice.

Bioassays

Tomiyama et al. (1998) studied β -adrenoreceptor subtypes mediating ureteral relaxation in rats, rabbits and dogs. β_3 -adrenoreceptor agonists were more effective in relaxing canine ureter than β_1 - and β_2 -adrenoreceptor agonists.

Koike et al. (1997) reported that the relaxant responses in the guinea pig taenia caecum are mediated by both the β_2 - and β_3 -adrenoreceptors.

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- Kalra et al. 1999) and in the gut. This includes peptides that are **orexigenic** (appetite-stimulating) signals and **anorectic** peptides. A review was given on investigational antiobesity agents and obesity therapeutic treatment targets by Bays (2004).

Neuropeptide Y (NPY), orexins A and B, galanin, melanin concentrating hormone (MCH), and agouti-related peptide (AgRP) all act to stimulate feeding, while **alpha-melanocyte stimulating hormone (α MSH)**, (see N.10.1), **corticotropin releasing hormone (CRH)**, (see N.9.4), **cholecystokinin (CCK)**, (see J.7.0.10), **cocaine and amphetamine regulated transcript (CART)**, **neurotensin, glucagon-like peptide 1 (GLP₁)**, (see K.4.0.3), **calcitonin gene related peptide (CGRP)**, (see N.10.3), **bombesin** (see J.3.1.8) and **ciliary neurotropic factor** (Xu et al. 1998) have anorectic actions (Tritos et al. 1999).

Mutations reducing the functional activity of leptin, the leptin receptor, α -MSH, and the melanocortin-4 receptors lead to obesity in animals. *Mc4r*-deficient (*Mc4r*^{-/-}) mice do not respond to the anorectic actions of MTH, an MSH-like antagonist, suggesting that α -MSH inhibits feeding primarily by activating *Mc4r* (Marsh et al. 1999).

Leptin (see L.4.1) is a 167 amino acid protein that is synthesized and secreted primarily by white adipose tissue, circulates in the blood and acts on receptors in the hypothalamus to decrease food intake and increase energy expenditure (Friedman and Halaas 1998; Trayhurn et al. 1999). Leptin and its derivatives are candidates for treatment of obesity, however, clinical studies (Considine et al. (1996) showed that most of obese humans have higher plasma levels of leptin than non-obese individuals, suggesting that obesity is associated with leptin resistance rather than leptin deficiency.

Neuropeptide Y (NPY) (see L.4.2) is a 36 amino acid peptide that is widely distributed throughout both the central and peripheral nervous systems and which plays a key role in the control of body weight. Central administration of NPY increases food intake (Stanley et al. 1992), while a reduction in endogenous neuropeptide Y leads to a decrease of food intake (Lambert et al. 1993). NPY antagonists are candidates for anti-obesity drugs.

Orexin-A and orexin-B (see L.4.3) are 33- and 28-residue peptides, also called hypocretins, which were originally isolated from rat hypothalamus (Sakurai et al. 1998). These peptides are located predominantly in the hypothalamus and locus coeruleus but are also found elsewhere in the brain. The orexins have a broad range of physiological functions, including the con-

L.4

Assays of Obesity-Regulating Peptide Hormones

L.4.0.1

Hormonal Regulation of Food Intake

Food intake and fat deposition are regulated by neurotransmitters peptides, most of the located in the brain, particularly in the hypothalamus (Elmqvist et al. 1999;

trol of feeding and energy metabolism. Food consumption is dose-dependently increased after intracerebroventricular infusion of orexin A and orexin B to rats (Jain et al. 2000). Orexin antagonists are potential anti-obesity drugs (Parker 1999).

Galanin (see L.4.4) is 29 amino acid C-terminally amidated peptide (30 amino acids in humans) which is localized mainly in the mammalian CNS, but also in other organs. Central administration of galanin increases and administration of galanin receptor antagonists (Crawley et al. 1990; Leibowitz and Kim 1992) decreases food intake. These data suggest the use of galanin receptor antagonists as anti-obesity agents.

Agouti-related protein affects pigmentation when its expression is limited to the skin, but ubiquitous expression causes obesity (Ollmann et al. 1997). The hypothalamic expression of agouti related protein is regulated by leptin, and overexpression of agouti related protein results in obesity and diabetes (Rosenfeld et al. 1998). Recombinant Agouti-related protein is a potent, selective antagonist at MC3R and MC4R, melanocortin receptor subtypes implicated in weight regulation (Fong et al. 1997; Shutter et al. 1997; Tota et al. 1999). Ubiquitous expression of human Agouti-related protein complementary DNA in transgenic mice caused obesity without altering pigmentation. Agouti-related protein is a neuropeptide implicated in the normal control of body weight downstream of leptin signaling. Ollmann et al. (1998) used a sensitive bioassay based on *Xenopus* melanophores to characterize pharmacological properties of recombinant Agouti protein.

Melanin concentrating hormone (MCH) is a cyclic 19 amino acid neuropeptide that was originally found to regulate pigmentation in fish. It plays a role in the central feeding behavior increasing food consumption (Qu et al. 1996; Rossi et al. 1999). Mice carrying a targeted deletion of the MCH gene are hypophagic and lean (Shimada et al. 1998). Among other factors, MCH may be a target of leptin signaling in the hypothalamus (Sahu 1998; Huang et al. 1999). Melanin-concentrating hormone is a functional melanocortin antagonist in the hypothalamus (Ludwig et al. 1998). MCH has been identified as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1) (Bacher et al. 1999; Chambers et al. 1999; Shimomura et al. 1999) which has been characterized by Saito et al. (1999). High binding capacity for MCH was found in human keratinocytes (Burgaud et al. (1997). Radioligands for the mammalian MCH receptor were described by Hintermann et al. (1999).

Cocaine- and amphetamine-regulated transcript (CART), a brain located peptide, has potent appetite suppressing activity and is closely associated with the actions of leptin and neuropeptide Y (Lambert et al. 1998; Kuhar and Dall-Vechia 1999). When injected intracerebroventricularly into rats, recombinant CART peptide inhibits both normal and starvation-induced feeding, and completely blocks the feeding response induced by neuropeptide Y (Kristensen et al. 1998). In the rat the CART gene encodes a peptide of either 129 or 116 amino acids whereas only the short form exists in humans (Thim et al. 1998a, b). A role of CART peptides in substance abuse and addiction is suggested by psychomotor-stimulant regulation of CART transcription in the striatum, as well as its localization within neural circuits that mediate reward and reinforcing behaviors (Couceyro and Lambert 1999). CART has been found not only in the hypothalamus and other brain areas (Broberger 1999; Koylu et al. 1998, 1999) but also in other organs, such as the pancreas (Jensen et al. 1999), in the rat sympatho-adrenal axis (Dun et al. 2000), or in the vagal afferent neurons sensitive to cholecystokinin (Broberger et al. 1999). CART can cross the blood-brain barrier (Kastin and Akerström 1999). CART receptor agonists are potential anti-obesity drugs (Couceyro and Lambert 1999).

Resistin

Steppan et al. (2001a), Berger (2001), Steppan and Lazar (2002, 2004), and Banerjee and Lazar (2004) showed that adipocytes secrete a signaling molecule with 114 amino acids in length which is called **resistin** (for resistance to insulin) and which links obesity to diabetes (Haluzik and Haluzikova (2006). Circulating resistin levels in mice are increased in diet-induced and genetic forms of obesity and are decreased by thiazolidindiones (see K.5.0.6). Central administration of resistin promotes short-term satiety in rats (Tovar et al. 2005). Administration of anti-resistin antibody improves blood sugar and insulin action in mice with diet-induced obesity. Treatment of normal mice with recombinant resistin impairs glucose tolerance and insulin action. Insulin-stimulated glucose uptake by adipocytes is enhanced by neutralization of resistin and is reduced by resistin treatment. Resistin with slightly modified amino acid sequence has also been found in humans.

Steppan et al. (2001b) described a family of tissue-specific **resistin-like-molecules (RELMs)**. **RELM α** is a secreted protein that has a restricted tissue distribution with highest levels in adipose tissue. Another family member, **RELM β** , is a secreted protein expressed

only in the gastrointestinal tract, particularly in the colon, in both mouse and human. *RELM β* gene expression is highest in proliferative epithelial cells and is markedly increased in tumors, suggesting a role in intestinal proliferation.

Banerjee et al. (2004) generated mice deficient in resistin by replacing the coding exons of the resistin gene (*rsn*) with the reporter gene *lacZ*. The mice exhibited low blood glucose levels after fasting due to reduced hepatic glucose production indicating a physiological function of resistin in maintenance of blood glucose during fasting. The lack of resistin diminished the increase in post-fast glucose associated with increased weight, suggesting a role for resistin in mediating hyperglycemia associated with obesity.

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L.4.1

Leptin

L.4.1.1

General Considerations on the Obese Gene Product Leptin

The obese gene product leptin is a cytokine-like non-glycosylated peptide of 146 amino acids that is secreted from adipose tissue. It is an important circulating hormone for the regulation of body weight (Arch and Beeley 1996; Hamann and Matthaei 1996; Friedman and Halaas 1998; Trayhurn 1999). MacDougald et al. (1995) described the expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes.

The mouse obese gene and its human homologue which show approximately 85% amino acid homology have been cloned by Zhang et al. (1994). Leptin binding was first reported in rat hypothalamic membranes (Stephens et al. (1995).

A **leptin receptor** was subsequently identified by expression cloning from a mouse chorioid plexus cDNA library (Tartaglia et al. 1995). Several transcripts of the leptin receptor as a result of alternative splicing which have different length of cytoplasmic region have been identified in mice and rats (Guan et al. 1997; Igel et al. 1997; Murakami et al. 1997) as well as leptin receptors in man (Maffei et al. 1995, 1996; Liu et al. 1997; Strosberg and Issad 1999). There are at least five different isoforms of the leptin receptor in mice. Mutations in the leptin receptor lead to massive obesity in *db* mice and *fa* rats (Friedman and Halaas 1998).

Administration of recombinant human leptin to mice increases thermogenesis and lipid oxidation in brown fat coupled with increased lipolysis and decreased fat synthesis in white and brown fat, which lead to a rapid reduction in the body weight (Sarmiento et al. 1997).

Leptin increases uncoupling protein expression in brown adipose tissue and oxygen consumption in mice (Scarpace et al. 1997).

Intracerebroventricular injection of recombinant murine leptin produces a dose-dependent reduction of laboratory diet in normal rats. This effect is attenuated in rats with diet-induced obesity (Widdowson et al. 1997).

The role of leptin in human obesity is controversial. Rare forms of human obesity have been identified where mutations in leptin or its receptor play a major role in the development of the disease. Leptin may be a signal for the onset of puberty in humans (Strosberg and Issad 1999). Leptin activates leptin-receptor expressing neurons in the arcuate region of the hypothalamus. Projections from these neurones stimulate melanocortin MC₄ receptors and neuropeptide Y-containing neurones to activate the sympathetic nervous system which controls lipolysis in white adipose tissue. The firing of gonadotrophin-releasing hormone-containing neurons and secretion of gonadotrophin-releasing hormone to the pituitary is elicited by leptin-mediated activation of leptin-receptor-expressing neurons and by other factors that control the reproductive axis (Chebab 2000).

Grasso et al. (1997, 1999) found inhibitory effects of leptin-related synthetic peptides on food intake and body weight gain after intraperitoneal administration in female C57BL/6*Job/ob* mice which may have potential application to the treatment of human obesity.

Ducy et al. (2000) and Takeda et al. (2002) suggest that leptin inhibits bone formation, probably via the sympathetic nervous system.

Dumond et al. (2003) showed in rats that leptin strongly stimulated anabolic functions of chondrocytes and induced the synthesis of IGF-1 and TGF β 1 in cartilage at both the messenger RNA and protein levels. A key role of leptin in osteoarthritis was assumed.

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- tion containing 7% sodium dodecyl sulfate, 50% formamide, 5× saline-sodium citrate buffer, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 50 mM sodium phosphate (pH 7.0) and 0.1% N-laurylsarcosine. Hybridization is performed at 42°C overnight in pre-hybridization solution containing oligonucleotide probe (25 ng/ml) specific to leptin, malic enzyme or 185 RNA. The following post-hybridization washes are performed: twice for 5 min in 2× saline-sodium citrate buffer/0.1% sodium dodecyl sulfate (at room temperature); twice for 15 min in 0.1× saline-sodium citrate buffer/0.1% sodium dodecyl sulfate (at 48°C). The membranes are then rinsed briefly with washing buffer containing 0.1 M maleic acid (pH 7.5), 0.15 M NaCl and 0.3% Tween 20. They are blocked by incubation for 30 min at room temperature with blocking buffer (1% blocking agent, 0.1 M maleic acid (pH 7.5), 0.15 M NaCl) and incubated (at the same conditions as described above) with blocking buffer containing a polyclonal antibody against digoxigenin conjugated to alkaline phosphatase. After washing twice for 15 min with washing buffer, the membranes are rinsed for 5 min with detection buffer (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl) and immersed for 5 min in CDP-Star solution (Boehringer Mannheim, Mannheim, Germany). Membranes are exposed to Kodak XAR film for 15 min to 1 h (Karbowska et al. 1999).

L.4.1.2

Determination of Leptin mRNA Level in Adipose Tissue

PURPOSE AND RATIONALE

Leptin mRNA levels in adipose tissue can be determined by Northern blot analysis (Friedrich et al. 1995; Harris et al. 1996; Zachwieja et al. 1997; Kochan et al. 1999).

PROCEDURE

Male Wistar rats weighing approximately 230 g treated for 14 days with test drug are sacrificed by decapitation. Liver, epididymal white adipose tissue and intracapsular brown adipose tissue are rapidly removed and frozen in liquid nitrogen. Total RNA is extracted from frozen tissues by a guanidinium thiocyanate-phenol/chloroform method (Chomczynski and Sacchi 1987). The RNA (10 µg per lane) is fractionated by horizontal gel electrophoresis. The RNA is transferred to a positively charged nylon membrane and fixed with UV-light. Pre-hybridization is performed at 42°C for 45 min in pre-hybridization solu-

EVALUATION

Signals are scanned and quantified using the NIH-Image software. The level of mRNA for leptin and malic enzyme as well as for 185 RNA is estimated using PeakFit software (Jandel Scientific). The values for leptin and malic enzyme mRNA are normalized to the corresponding amount of 185 RNA. Results expressed in arbitrary units are presented as means ± SEM of samples of 10 rats. The statistical difference between groups is assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or by Mann-Whitney test.

MODIFICATIONS OF THE METHOD

Richards et al. (2000) described quantitative analysis of leptin mRNA using competitive reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection.

Shintani et al. (2000) determined leptin mRNA levels in primary cultured rat adipocytes.

Li et al. (1999) used an anti-leptin polyclonal antiserum to detect leptin-immunoreactivity in the central nervous system in rats.

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L.4.1.3**Determination of Plasma Leptin****PURPOSE AND RATIONALE**

Plasma leptin levels are determined by radioimmunoassay using a rat-specific antibody (Ciba-Geigy, Basel, Switzerland) (Zachwieja et al. 1997) or with a double-antibody radioimmunoassay kit based on a mouse standard (Linco Research, St. Louis, MO) (Surwit et al. 1997; Van Heek et al. 1997). A radioimmunoassay for leptin in human plasma was described McGregor et al. (1996).

PROCEDURE

The peptide VPIQKVQDDTKLIKTIVT representing the first twenty amino acids of the predicted sequence of mature leptin protein (leptin 1–20) was synthesized with 9-fluorenyl methyl oxycarbonyl-(Fmoc)-protected L-amino acids on a applied Biosystems peptide synthesizer and purified by HPLC on a Dynamax column developed with a 0.1% trifluoroacetic acid (TFA)/H₂O/acetonitrile (8% to 40%) gradient.

The peptide is conjugated to thyroglobulin using carbodiimide as coupling reagent. Rabbits are immunized by intradermal injections and boosted at 4 weeks intervals.

A peptide identical to the one used for antibody generation except for a tyrosine residue added to the C-terminal end is labelled with ¹²⁵I using the oxidizing agent Iodogen (Pierce, USA) and purified on HPLC. For radioimmunoassay, the buffer consists of Tris-HCl 1 M, pH 7.4; 0.1% gelatin; 0.1% Triton X-100, and 0.01% NaN₃. Unlabelled *ob* peptide is used for standards. 100 μl of standard or unknown, 200 μl buffer, 100 μl antibody at a final concentration of 1:80000 and 100 μl ¹²⁵I-leptin peptide (approximately 5000 cpm) are added to polystyrene tubes and incubated for 48 h at 4°C. Antibody-bound and unbound peptide are separated by addition of goat anti-rabbit immunoglobulin antibody followed by centrifugation. The precipitates are counted on a computer-linked gamma-counter and the leptin concentrations of the samples are obtained using the “RIA-Calc” program.

EVALUATION

Results are expressed as means ± SD, and statistical comparisons are made with the use of Student's *t*-test. Linear regression analysis is performed with the method of least squares.

MODIFICATIONS OF THE METHOD

Maffei et al. (1995) used an immunoprecipitation assay to measure plasma leptin levels. A quantitative increase of the signal intensity was seen on Western blots when increasing amounts of recombinant protein were added to *ob* mouse serum, which does not have leptin. Densitometry was used to compare the signal intensity of the native protein in mouse plasma to the standard. The level of leptin-like immunoreactivity was quantified as nanograms per ml.

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L.4.2

Neuropeptide Y

L.4.2.1

General Considerations on Neuropeptide Y and Related Peptides

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) are endogenous 36-amino acid peptides belonging to the same family, which is characterized by a tertiary structure termed 'PP-fold' (Larhammar 1996; Berglund et al. 2003). They have different tissue distributions (Sundler et al. 1993) and act in different physiological systems, mostly neuronal for NPY and endocrine for PYY and PP (Wettstein et al. 1995; Playford and Cox 1996; Goumain et al. 1998).

Neuropeptide Y plays a key role in the control of body weight (Leibowitz 1994; Kalra and Kalra 2004). Central administration of neuropeptide Y increases food intake and decreases thermogenesis in satiated animals (Billington et al. (1991, Stanley et al. 1992), while a reduction in endogenous neuropeptide Y leads to a decrease of food intake (Lambert et al. 1993). Hypothalamic neuropeptide Y and mRNA levels are increased after fasting and in genetically obese mice (Stephens et al. 1995). Neuropeptide Y is required for the maintenance of the obese phenotype of the leptin-deficient *ob/ob* mice (Erickson et al. 1996). Conversely, leptin appears to decrease food intake and body weight in part by decreasing neuropeptide Y synthesis and release (Stephens et al. 1995). These data suggest that neuropeptide Y is a key modulator of body weight and that neuropeptide Y receptor antagonists may be useful anti-obesity agents.

Neuropeptide Y mediates its physiological effects via interaction with at least six distinct G protein-coupled receptors, designated Y₁, Y₂, Y₃, Y₄, Y₅ and Y₆. (Marsh et al. 1998; Michel et al. 1998; Wyss et al. 1998; Iyengar et al. 1999; Larhammer and Salanek 2004; Lin et al. 2004).

Central administration of peptide analogues of neuropeptide Y has been used to study the physiological roles of neuropeptide Y, especially the role of the peptide in feeding behavior. Kanatani et al. (1999) demonstrated an anorexigenic effect against NPY induced feeding in rats by an Y₁ antagonist with high selec-

tivity and potency for the Y₁ receptor. Central administration of peptides that activate the Y₅ receptor, peptide YY (PYY), PYY(3–36), [Leu³¹,Pro³⁴]NPY, and human pancreatic polypeptide elicit feeding, whereas central administration of central administration of peptides that are inactive at the Y₅ receptor do not elicit feeding (Gerald et al. 1996). Fuhlendorff et al. (1990) described [Leu³¹, Pro³⁴]neuropeptide Y as a specific Y₁ receptor agonist. While other neuropeptide Y analogues or fragments interacts with several neuropeptide Y receptors, [D-Trp³²]NPY is a completely selective Y₅ agonist that elicits feeding at relatively high doses (Gerald et al. 1996). Peptides with activity on the Y₁ receptor produce conflicting effects on feeding. DesAA^{10–17}[Cys^{7–21},Pro³⁴]NPY is a Y₁ receptor agonist with a potency equivalent to neuropeptide Y, but does not elicit feeding after central administration (Kirby et al. 1995).

Schaffhauser et al. (1997) described inhibition of food intake after intracerebroventricular injection of neuropeptide Y Y₅ receptor antisense oligodeoxynucleotides in rats.

Dumont et al. (2004) recommended receptor autoradiography as means to explore the possible functional relevance of neuropeptides such as neuropeptide Y.

Criscione et al. (1998) reported inhibition of food intake in rats by an Y₅ receptor antagonist under various conditions. Several neuropeptide Y antagonists were synthesized which inhibit food intake in rodents and may be used as anorectic agents (Matthews et al. 1997; Kask et al. 1998; Parker et al. 1998; Shigeri et al. 1998; Wieland et al. 1998; Zarrinmayeh et al. 1998).

Besides the stimulation of feeding, NPY and PYY show several other activities: influence on gastric motility (Chen et al. 1997), antisecretory effects in the gastrointestinal tract (Souli et al. 1997), anti-stress activity (Heilig 2004), anti-epileptic activity (Baraban 2004; Vezzani and Sperk 2004), central and peripheral control of the cardiovascular system (Lew et al. 1996; Tadepalli et al. 1996; Hudspith and Muglani 1997; McCloskey et al. 1997), effects on renal function (Blaze et al. 1997; Bischoff et al. 1997), antinociceptive effects (Broqua et al. 1996), or release of ACTH (Small et al. 1997). Kask et al. (2001) described the effects of a neuropeptide Y Y₅ receptor antagonist on food intake and anxiety-related behavior in the rat.

Sajdyk et al. (1999) found that amygdalar neuropeptide Y Y₁ receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test, whereas neuropeptide Y-Y₂ receptors mediate anxiety in the amygdala (Sajdyk et al. 2002).

Neuropeptide Y plays a role in neurobiological responses to ethanol and drugs of abuse (Thiele et al. 2004).

NPY receptor agonists show anxiolytic effects in rat conflict tests (Britton et al. 1997; Kask et al. 2002); neuropeptide Y₁ antagonists have anxiogenic-like effects (Kask et al. 1996).

Goumain et al. (1998) generated a rat Y₅ clone by reverse transcription from rat brain, polymerase chain reaction and transfection to COS-7 cells. Isolated jejunal crypt and villus cells and colon epithelial cells were analyzed for the Y₅ receptor by reverse transcription and polymerase chain reaction. Inhibition of binding of [¹²⁵I]peptide YY in these cells by various peptides was studied.

Mullins et al. (2000) characterized the cloned neuropeptide Y₆ receptor in mice.

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L.4.2.2

Receptor Assay of Neuropeptide Y

PURPOSE AND RATIONALE

At least six distinct G protein-coupled receptors of neuropeptide Y have been identified, designated Y₁, Y₂, Y₃, Y₄, Y₅ and Y₆ (Doods and Krause 1991; Marsh et al. 1998; Michel et al. 1998; Bischoff and Michel 1999).

Sheikh et al. (1989) described a receptor binding assay for neuropeptide Y.

PROCEDURE

¹²⁵I-Labeling

¹²⁵I-labeled NPY is prepared using carrier-free Na¹²⁵I and 1,3,4,6-tetrachloro-3 α ,6-diphenylglycouril (Ser-

va Heidelberg, Germany) as oxidizing agent. Synthetic porcine NPY (7 nmol) in 40 μ l of sodium phosphate buffer (pH 7.4, 0.1 M) and 1 mCi of Na¹²⁵I in 10 μ l of dilute NaOH is added to a reaction vial which has been coated with 20 μ g of chlorglycouril by evaporation of 20 μ l of a solution of glycouril in methylene chloride. Following incubation in ice for 5 min, 50 μ l of 35% acetonitrile is added prior to HPLC.

Purification by Reverse Phase-High Performance Liquid Chromatography

A prepacked Nucleosil 300–5 C₁₈ cartridge is used. The chromatography is performed at 50°C with a flow rate of 1 ml 35% acetonitrile/min. Fractions of 0.5 ml are collected into tubes containing 0.1 ml of acetic acid (0.5 M) containing 10 mg/liter bovine serum albumin. Aliquots of 10 μ l are counted in a γ -counter, and fractions containing the radiolabeled peptide are pooled and stored at –20°C.

Preparation of Synaptosomal Membranes

Synaptosomal membranes are prepared from the hippocampus of Danish LYY-strain landrace/Yorkshire pigs. The tissue is homogenized in 0.32 M sucrose in a Sorval Omni-Mixer at 0°C for 5 min, followed by centrifugation (1000 g, 10 min). The supernatant is removed and centrifuged (20,000 g, 30 min) to form the crude mitochondrial fraction. After resuspension in 3 ml of sucrose, this fraction is subfractionated on a discontinuous density gradient consisting of 0.8 M and 1.2 M sucrose (3 ml of each) in a SW 27 rotor and a Beckman ultracentrifuge (100,000 g, 90 min). The synaptosomes which concentrate at the interface between the two sucrose concentrations are collected and recentrifuged (50,000 g, 10 min). The resulting pellet is washed with HEPES buffer (pH 7.4, 25 mM) containing CaCl₂ (2.5 mM) and MgCl₂ (1 mM), centrifuged (50,000 g, 10 min), and resuspended in HEPES buffer. The protein concentration is determined using the Bio-Rad protein assay with bovine serum albumin as standard. The membrane preparation is diluted in binding buffer to a protein concentration of 2 g/liter and stored at –80°C.

Receptor Binding Assay

The binding buffer is a HEPES buffer (pH 7.4, 25 mM) containing CaCl₂ (2.5 mM) and MgCl₂ (1 mM), bovine serum albumin (10 g/liter), and bacitracin (0.5 g/liter). The incubation mixture consists of 0.5 ml of membrane suspension, diluted with binding buffer to a protein concentration of 200 mg/liter, 0.05 ml of

unlabeled peptide, and 0.5 ml of ¹²⁵I-labeled peptide (40,000 cpm). After 1 h of incubation, triplicate aliquots of the incubation medium are transferred to polypropylene tubes (0.4 × 4.5 cm), containing 200 μ l of ice-cold buffer. Membrane-bound, radiolabeled peptide is separated from the free peptide by centrifugation (7500 g, 2 min) in a Beckman Microfuge B. The supernatant is aspirated, and the tube and pellet are gently washed with buffer (0.5 ml). The tip of the tube is cut off and counted in the γ -counter.

EVALUATION

The specific binding is calculated as the difference between the amount of ¹²⁵I-labeled peptide bound in the absence (total binding) and presence (nonspecific binding) if 10 μ M unlabeled peptide.

The concentration dependence of the receptor binding is determined by incubating the membranes with increasing concentrations of radiolabeled peptide in the absence or presence of unlabeled peptide.

MODIFICATIONS OF THE METHOD

Rose et al. (1995) reported cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor.

Gehlert et al. (1996) recommended [¹²⁵I][Leu³¹, Pro³⁴]peptide YY as selective radioligand for the neuropeptide Y Y₁ receptor and for human pancreatic polypeptide 1 receptors.

Robin-Jagerschmidt et al. (1998) investigated the ligand binding site of neuropeptide Y at the rat Y₁ receptor by construction of mutant receptors and [³H]NPY binding studies.

Dumont et al. (1998) investigated the respective distribution of neuropeptide Y Y₁, Y₂, Y₄, and Y₅ receptor subtypes in rodents (rat and mouse), guinea pig, and primates (marmoset and vervet monkey and human) brains, representing three orders of mammals.

Primus et al. (1998) measured guanyl 5'(γ [³⁵S]-thio)-triphosphate binding to NPY receptor-activated G-proteins in adult rat brain sections in order to determine the neuroanatomical distribution of NPY receptor subtypes.

Wyss et al. (1998) administered various doses of synthetic neuropeptide Y agonists intracerebroventricularly to rats in order to establish dose-response curves and to estimate ED₅₀ values of feeding. These values were compared with binding affinities (IC₅₀) for rat NPY receptor subtypes Y₁, Y₂, Y₄, and Y₅ *in vitro*. Mouse fibroblast cell lines (LMTK–) were stably transfected with the rat Y₁, Y₂ and the Y₄ recep-

tor, whereas human embryonic kidney HEK-293 cells were used for transfection with the rat Y₅ receptor.

Parker et al. (1998) determined the agonist and antagonist potency of neuropeptide Y and various analogues for cloned human and rat neuropeptide Y receptors expressed in CHO or 293 cells.

Wieland et al. (1998) studied the subtype selectivity of a nonpeptide Y₁ receptor antagonist using membranes from rat hypothalamus, from SK-N-MC (neuroblastoma) cells and from SMS-KAN (neuroblastoma) cells; human Y₁ receptor stably expressed in baby hamster kidney (BHK) cells, rat Y₁ receptor expressing human embryonic kidney (HEK) 293 cells, human Y₂ receptor stably expressed in BHK cells, rat Y₄ stably transfected in BHK cells, and human Y₅ receptor stably transfected in HEK 293 cells.

Several groups reported screening results of neuropeptide Y antagonists mostly using receptor binding experiments (Murakami et al. 1999; Fotsch et al. 2001; Islam et al. 2002; Poindexter et al. 2002; Andres et al. 2003; Hammond et al. 2003; Blum et al. 2004; Gillman et al. 2006; Kakui et al. 2006).

Dautzenberg et al. (2005) established robust functional assays for the characterization of neuropeptide Y receptors. The human neuropeptide Y (NPY) receptors 1 (hY₁), 2 (hY₂), 4 (hY₄), and the mouse type 5 (mY₅) receptor were expressed in human embryonic kidney 293 (HEK293) cells. The receptors bound a radioiodinated NPY ligand with high affinity and various NPY analogs competed for binding in a receptor-selective manner. Similarly, cAMP inhibition and GTP γ S binding assays were established. The four NPY receptors were further tested in the fluorimetric imaging plate reader (FLIPR) format, a cellular high-throughput assay, in the absence and presence of chimeric G proteins, Gq₀₅, Gq_{i5} and Gq_{i9}.

PROCEDURE

Cell Transfections

cDNAs of the four NPY receptors inserted into pcDNA3 (5 μ g each) or Gq_{i5}, Gq₀₅ and Gq_{i9} (10 μ g each) inserted into pcDNA5 (Invitrogen) were transiently transfected into HEK293 cells with the FUGENE reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. Cell lines stably expressing the various NPY receptors together with Gq_{i9} were generated by transfecting receptor cDNA (0.5 μ g each), the Gq_{i9} cDNA (1 μ g) and the pOG44 vector (10 μ g) encoding the cDNA for the DNA recombinase used in the Flip-in system (Invitrogen) with the FUGENE reagent. Two days after transfection, Geneticin (500 μ g/ml) and hy-

gromycin (50 μ g/ml) selection was initiated and stable cell clones robustly responding in the FLIPR assay were selected.

Radioreceptor Binding Assays

Membranes from NPY-receptor-expressing HEK cells were prepared according to Dautzenberg et al. 2005. Competition binding assays were performed with 5 (hY₂ or hY₄), 25 (mY₅) and 40 μ g (hY₁) of membrane proteins prepared from the four NPY receptors under assay conditions according to Rist et al. (1996), using 96-well plates (Beckmann Instruments, Fullerton, Calif., USA) and a scintillation proximity assay (SPA; Dautzenberg et al. 2000). The membranes, 1 mg wheat germ agglutinin SPA beads (Amersham Pharmacia Biotech), 100 pM [¹²⁵I]PYY and unlabeled peptides (10⁻⁵ M to 10⁻¹¹ M) were added. The reaction mixture was incubated on a shaker for 120 min at 22°C and then read in a TopCount (Packard). Non-specific binding was determined as residual binding in the presence of 10 μ M PYY. The dissociation constant K_d, the inhibition constant K_i, and the maximum receptor concentration, B_{max}, were calculated using the interactive curve fitting program Xlfit (Hoffmann-La Roche). Under these conditions less than 10% of the total radioactivity was specifically bound by the various receptor constructs.

cAMP Inhibition Assay

The inhibition of forskolin-mediated cAMP accumulation by NPY analogs in 96-well plates was determined as previously described (Dautzenberg et al. 2001). Briefly 20,000 cells were incubated in Krebs-Ringer-HEPES-buffered solution (KHR; 124 mM NaCl, 5 mM KCl, 1.25 mM MgSO₄, 1.5 mM CaCl₂, 1.25 mM KH₂PO₄, 25 mM HEPES, pH 7.4) in the presence of 1 μ M forskolin (Sigma, Munich, Germany) with increasing concentrations of agonists (10 pM to 10 μ M) for 60 min at 37°C. Reactions were stopped by the addition of 0.12 ml ice-cold ethanol and stored at -80°C for at least 4 h. The cAMP content was determined from the supernatant using the Biotrak non-radioactive cAMP kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

GTP γ S Binding Assay

Agonist-mediated binding of GTP γ ³⁵S was investigated in the SPA format using 96-well plates (Rover et al. 2000) and membranes prepared from cells transfected with the hY₁, hY₂, hY₄ and mY₅ receptors. Binding was performed in 200 μ l of 20 mM HEPES-buffer (pH 7.4, plus 6 mM MgCl₂ and 100 mM NaCl),

supplemented with 20 μ M GDP, 10 μ M cold GTP γ S and 0.3 nM GTP γ ³⁵S. Then, 20 μ g (hY₂, hY₄, mY₅) to 40 μ g (hY₁) membranes, 1 mg wheatgerm agglutinin SPA beads, NPY analogs or synthetic compounds (10⁻⁴ M to 10⁻¹¹ M) were added. For antagonist assays, a submaximal (EC₈₀) concentration of PYY was added together with increasing antagonist concentrations (10⁻⁴ to 10⁻¹¹ M). The reaction mixture was incubated on a shaker for 60 min at 22°C and then centrifuged for 5 min at 1500 rpm in an Eppendorf 5403 centrifuge. Finally the plates were read in a TopCount reader (Packard).

Calcium Mobilization Assays

HEK293 transiently stably expressing the four NPY receptors with or without chimeric G-proteins were seeded at a density of 100,000 cells into poly-D-lysine-coated 96-well blackwall, clear-bottom microtiter plates (Corning, NY) (Dautzenberg et al. 2004). One day later, the medium was removed and 100 μ l loading medium [DMEM high glucose, without serum, supplemented with 10 mM HEPES acid, 0.1% BSA, 5 mM probenecid and 2 μ M Fluo-3AM (Molecular Probes, Leiden, The Netherlands)] was added. Cells were loaded for 1 h at 37°C, washed twice with 100 μ l assay buffer (5 mM HEPES acid, 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, 10 mM glucose) and then 150 μ l assay buffer was added. Cells were further preincubated at room temperature before adding agonists or agonists plus antagonists in 50 μ l assay buffer and then measured on a T-channel fluorimetric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, Calif., USA). Maximum change in fluorescence over baseline was used to determine agonist response.

EVALUATION

Statistical analyses of the binding, cAMP, GTP γ S and calcium mobilization data were performed on a Macintosh PC using StatView software (SAS, Cary, N.C., USA) by one-way analyses of variance (ANOVAs) across experimental groups followed by the Dunnett's test with the alpha set at 0.05.

Beauverger et al. (2005) characterized antagonists specific to human neuropeptide Y receptor subtype 5 using a **luciferase reporter gene assay**.

PROCEDURE

CRE-Luciferase Reporter Assay

Standardization of this assay consisted of testing various cell densities and incubation times to optimize the signal-to-background ratio. Briefly, HEK293-hMT1 cells grown to \approx 80% confluence were transfected in

batches with lipofectAMINE Plus (Life Technologies) using 20 ng of pcDOR8, 500 ng of p Δ MC16-Luc and 1 μ g of either pcDNA3.1 or pcDNA3.1-Y5 per million of cells. Then, 24 h after transfection, cells were detached by agitation, centrifuged and recovered at 10⁶ cells/ml in phenol-red-free DMEM medium supplemented with 2% fetal calf serum. They were then seeded as 50- μ l aliquots into white 96-well tissue culture plates containing the drugs previously diluted in the same medium and distributed as 50- μ l aliquots. After a 20-h incubation period, the luciferase activity was measured by adding 100 μ l per well of Lucifer buffer (Packard) followed by a 30-min incubation in the dark at room temperature and a use of a Topcount (Packard).

Savontaus et al. (1998) measured expression of preproneuropeptide Y mRNA in the arcuate nucleus and preprocorticotropin-releasing factor mRNA in the paraventricular nucleus by *in situ* hybridization technique after short and long-term treatment with a β ₃-adrenoreceptor agonist.

Bioassays have been used for characterization and classification of neuropeptide Y receptors (Hedge et al. 1995; Pheng and Regoli 1998; Dumont et al. 2000a, b). Some isolated organs appear to be "monoreceptor" systems, e. g., the rat tail artery (Gicquiaux et al. 1996), the rabbit saphenous vein (Pheng and Regoli 1998; Feletou et al. 1999) or the isolated perfused kidney of the rat (Hedge et al. 1995) for the Y₁ receptor. The dog saphenous vein, the rat vas deferens and the rat colon can be used for the Y₂ receptor, the rat colon for the Y₄ receptor, and the rabbit ileum for the Y₅ receptor (Pheng et al. 1997).

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L.4.3

Orexin

L.4.3.1

General Considerations on Orexin

Orexin-A and orexin-B are 33- and 28-residue peptides, also called hypocretins, which were originally isolated from rat hypothalamus (De Lecea et al. 1998; Sakurai et al. 1998). Both peptides are derived from a 130 amino acid precursor, preproorexin, which is encoded by a gene localized to chromosome 17q21 in humans. These peptides are located predominantly in the hypothalamus and locus coeruleus (Evans et al. 1999), but are also found elsewhere in the brain, and in the spinal cord (Smart 1999; Van den Pol 1999). The orexins have a broad range of physiological functions, including the control of feeding and energy metabolism (Dube et al. 1999; Mondal et al. 1999; Sakurai 1999, 2006), stimulation of insulin secretion (Nowak et al. 2000, 2005), modulation of neuro-endocrine function (Date et al. 1999), regulation of the sleep-wake cycle (Piper et al. 2000), narcolepsy (Taheri et al. 2002; Wille et al. 2003; Mieda et al. 2004), stress and anxiety, behavioral activities (Ida et al. 1999; Siegel 2003), cardiovascular (Shirasaka et al. 1999; Chen et al. 2000), adrenal function (Jöhren et al. 2004), and sexual and reproductive functions (Pu et al. 1998; deLecea and Sutcliffe 1999; Tamura et al. 1999). Food consumption is dose-dependently increased after intracerebroventricular infusion of orexin A and orexin B to rats which can be suppressed by a NPY Y_1 antagonist (Jain et al. 2000). Food intake could be inhibited by central injection of an anti-orexin antibody in fasted rats (Yamada et al. 2000). Novak et al. (2000) found an increase of insulin secretion after subcutaneous injection of orexins to rats.

Orexin antagonists are potential anti-obesity drugs (Parker 1999).

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L.4.3.2

Receptor Assay of Orexin

PURPOSE AND RATIONALE

The peptides orexin A and orexin B bind both to two receptors, orexin-1 (OX₁) and orexin-2 (OX₂), although orexin B has a low affinity for OX₁ (Sakurai et al. 1998). Differential expression of orexin receptor 1 and 2 in rat brain was reported by Marcus et al. (2001) and distinct recognition of OX₁ and OX₂ receptors by orexin peptides by Ammoun et al. (2005).

PROCEDURE

Orexin OX₁ and OX₂ receptors are produced by polymerase chain reaction from foetal and adult brain cDNA libraries, using primers located across the start and stop codons. The receptors are subcloned into the pCDN vector (with neomycin resistance) and transfected into CHO cells using lipofectamine (Life Technologies). Clones are selected using 400 µg/ml G418 (Life Technologies) and single clones are produced by limiting dilution cloning.

CHO-OX₁ and CHO-OX₂ cells are routinely grown as monolayers in MEM-Alpha medium supplemented with 10% fetal calf serum and 400 µg/ml G418 and maintained under 95% O₂/5% CO₂ at 37°C. Cells are passaged every 3–4 days.

Synthetic human orexin A is ¹²⁵I-labeled at Tyr¹⁷ by chloramine-T oxidation in the presence of Na¹²⁵I (2000 Ci/mmol). Monoiodinated peptide is purified by C¹⁸ reverse-phase HPLC (Takigawa et al. 1995). Stable transfectant CHO cell lines expressing human OX₁R or OX₂R are each seeded onto 12-well plates at a density of 3 × 10³ cells per well. After an overnight culture, medium is discarded and cells are incubated at 20°C for 90 min with binding buffer (HEPES-buffered saline/5% bovine serum albumin) containing 10⁻¹⁰ M [¹²⁵I]orexin A plus designated concentrations of unlabeled competitors. Cells are then washed three times with ice-cold phosphate-buffered saline and lysed in 0.1 N NaOH. Cell-bound radioactivity is determined by a γ-counter.

EVALUATION

Data are expressed as the percentage of saturably bound reactivity in the absence of nonradioactive peptide. For each experiment each value is determined in duplicate, and the results are expressed as the means ± standard errors of at least 3 separate experiments.

MODIFICATIONS OF THE METHOD

Smart et al. (1999) studied the pharmacology of recombinant orexin receptors using the fluorescence imaging plate recorder (FLIPR). CHO-OX₁ or CHO-OX₂ cells were seeded into black walled clear base 96 well plates at a density of 20000 cells per well in MEM-Alpha medium supplemented with 10% fetal calf serum and 400 µg/ml G418 and cultured overnight. The cells were then incubated with MEM-Alpha medium containing the cytoplasmic calcium indicator, Fluo-3AM (4 µM) and 2.5 mM probenecid at 37°C for 60 min. The cells were washed four times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 1% gelatin, be-

fore being incubated for 30 min at 37°C with either buffer alone (control) or buffer containing various signal transduction modifying agents. The plates were then placed into an FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence ($\lambda = 488 \text{ nm}$, $\lambda = 540 \text{ nm}$) (Sullivan et al. 1999) before and after the addition of orexin A or orexin B.

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L.4.3.3

Radioimmunoassay for Orexin

PURPOSE AND RATIONALE

A radioimmunoassay for orexin A has been developed by Mitsuma et al. (2000).

PROCEDURE

Synthetic orexin A is conjugated on an equal weight basis to BSA using glutaraldehyde. New Zealand white rabbits are immunized with an emulsion of this conjugate in one ml of water and complete Freund's adjuvant (1:2, v/v) which is injected into the foot pad at intervals of 3 weeks. Blood is withdrawn one week after each injection, and the presence of anti-orexin is checked by radioimmunoassay.

Radioiodination of orexin A is performed with the chloramine T method. The radioiodinated materials are chromatographed on Sephadex G-25, eluted with

0.01 M phosphate buffer (pH 7.4), and collected in 1.0 ml fractions. The first peak is orexin A- I^{125} .

For determination of tissue concentrations, brain tissues of rats are dissected into the hypothalamus, cerebral cortex, thalamus, striatum, hippocampal formation, brain stem and cerebellum.

For the extraction of orexin A, the freshly obtained tissues are weighed and placed in 5.0 ml acid-acetone, homogenized and centrifuged.

For the double antibody radioimmunoassay, 0.1 ml of standard or samples, 0.1 ml of antibody (1:1000), 0.1 ml orexin A I^{125} , and 0.5 ml buffer are incubated for 24 h at 4°C. Then 0.1 ml of the second antibody solution is added and incubated again for 24 h at 4°C. The samples are centrifuged, the supernatants decanted and radioactivity counted in the precipitates.

EVALUATION

Bound/total count is calculated and standard curves versus synthetic orexin A are established.

MODIFICATIONS OF THE METHOD

Using separate radioimmunoassays for orexin A and orexin B, Mondal et al. (1999) determined their distribution in microdissected nuclei of the diencephalon and brainstem of rats.

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L.4.4

Galanin

L.4.4.1

General Considerations on Galanin

Galanin is a neuropeptide of 29 amino acids in length (30 amino acids in humans) which was first isolated from porcine intestine (Tatemoto et al. 1983) and is localized mainly in the mammalian CNS (Skofitsch and

Jacobowitz 1985; Melander et al. 1986), but also in other organs (Baltazar et al. 2000). Central administration of galanin increases food intake in satiated rats (Crawley et al. 1990). Conversely, reduction of central galanin levels by antisense oligonucleotide techniques (Akabayashi et al. 1994) or central administration of galanin receptor antagonists (Leibowitz and Kim 1992) decreases food intake. The activity of galanin in the hypothalamus is modulated by metabolic hormones and by the ingestion of nutrients (Wang et al. 1998).

These data suggest the use of galanin receptor antagonists as anti-obesity agents. More recent studies concentrate on pain and CNS effects (Kask et al. 1997; Holmes et al. 2000; O'Meara et al. 2000; Xu et al. 2000; Counts et al. 2003; Elliott-Hunt et al. 2004; Krasnow et al. 2004; Badie-Mahdavi et al. 2005a; Lundström et al. 2005).

Physiological actions of peripherally administered galanin include contraction of smooth muscle (Ekblad et al. 1989; Ahtaridis et al. 1998; Korolkiewicz et al. 1998; Niiro et al. 1998), inhibition of glucose-stimulated insulin release (McDonald et al. 1985; Leonhardt et al. 1989), influence on learning and memory behavior (McDonald and Crawley 1997; McDonald et al. 1998a, b; Gleason et al. 1999; ögren et al. 1999; Zachariou et al. 1999), antinociceptive activity in rats with experimentally induced neuropathy (Burazin and Gundlach 1998; Ma and Bisby 1999; Yu et al. 1999; Kerr et al. 2000; Wang et al. 2000). Based on pharmacological and molecular biological evidence, Mazarati et al. (2001) discussed the hypothesis that galanin works as an endogenous anticonvulsant. A nonpeptide galanin receptor agonist showed anticonvulsant activity (Shi et al. 2002).

Galanin-overexpressing transgenic mice showed no anxiety-related behaviors in three different tests (Holmes et al. 2002), whereas galanin GAL-R1 receptor null mutant mice displayed increased anxiety-like behavior (Holmes et al. 2003).

Galanin has been found to influence secretion of growth hormone (Ottlecz et al. 1988; Murakami et al. 1987), LH (Todd et al. 1998), luteinizing hormone (Finn et al. 1998), and prolactin (Koshiyama et al. 1987; Cai et al. 1998; Wynick et al. 1998), to inhibit dopamine release from the median eminence (Nordström et al. 1987), to influence ACh release from rat brain (Fisone et al. 1987; Kasa et al. 1998), to inhibit norepinephrine release from the hypothalamus (Tsuda et al. (1989) and to modulate 5-HT_{1A} receptors in the ventral limbic cortex of the rat (Diaz-Cabiale et al. 2000).

A fragment of the galanin precursor protein, galanin message-associated peptide (GMAP), is present in dorsal root ganglion cells and influences the spinal nociceptor flexor reflex in rats (Xu et al. 1996).

A galanin-like peptide, named GALP, was isolated by Ohtaki et al. (1999) from porcine hypothalamus.

Some synthetic **galanin receptor agonists** were described, such as galnon (Saar et al. 2002; Bahdie-Mahdavi et al. 2005b) or galmic (Ceide et al. 2004; Bartfai et al. 2004).

Various **galanin receptor antagonists**, such as galantide (Lindskog et al. 1992; Sahu et al. 1994; Arletti et al. 1997; Ceresini et al. 1998), and other compounds (Bartfai et al. 1991; Pramanik and ögren 1992; Kask et al. 1995; Xu et al. 1995; Kakuyama et al. 1997; Pooga et al. 1998; Koegler et al. 1999; Park and Baum 1999; Katoh and Ohmori 2000; Kisfalvi et al. 2000; Swanson et al. 2005; ögren et al. 2005) were synthesized and tested.

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L.4.4.2

Receptor Assay of Galanin

PURPOSE AND RATIONALE

Galanin mediates its physiological effects via interaction with at least three G protein-coupled receptors, designated GAL1, GAL2, and GAL3 receptor (Wang and Parker 1998; Iismaa and Shine 1999; Branchek et al. 2000; Waters and Krause 2000).

The first known galanin receptor GAL1 has been isolated from the human Bowes melanoma cell line (Habert-Ortoli 1994). Human GAL1 contains 349 amino acids with the structure of a G protein-coupled receptor. A rat GAL1 homologue, cloned from Rin14B cells, contains 346 amino acids (Parker et al. 1995). Human GAL1 mRNA has been detected by northern blot analysis in fetal brain and small intestinal tissue, and also by reverse transcriptase-polymerase chain reaction in the human gastrointestinal tract from

the esophagus to the rectum. Rat GAL1 mRNA has been detected by northern blot analysis in the brain, spinal cord and Rin14B cells. Human and rat GAL1 share similar binding profiles in [¹²⁵I]galanin binding assays (Sullivan et al. 1997).

Heuillet et al. (1994) described ligand binding and functional characteristics of the human galanin receptor in the Bowes melanoma cell line.

Jungnickel and Gundlach (2005) studied [¹²⁵I]-galanin binding in brain of wild-type, and galanin- and GALR1-knockout mice.

The rat GAL2 receptor has been cloned and characterized by Wang et al. (1997), Ahmad et al. (1998), the mouse GAL2 receptor by Pang et al. (1998) and the human GAL2 receptor by Bloomquist et al. (1998) and Fathi et al. (1998). Unlike GAL1, mRNA encoding rat GAL2 is widely distributed in all tissues examined including the brain and peripheral tissues. Likewise, the human GAL2 receptor is detectable by RT-PCR in several central and peripheral tissues. GalR2 plays a key role in neurite outgrowth from adult sensory neurons (Mahoney et al. 2003). Rat GAL1 and GAL2 share similar pharmacological profiles in that they possess high affinity for full-length and N-terminal fragments of galanin.

Rat and human GAL3 receptors were described by Kolakowski et al. (1998), Smith et al. (1998). Human and rat GAL3 share similar profiles in [¹²⁵I]galanin receptor binding assays.

Lee et al. (1999) reported the isolation of a cDNA clone named GPR54 which encodes a G-protein coupled receptor related to the galanin receptors.

PROCEDURE

Preparation of the radioligand ¹²⁵I-galanin is performed by iodination of galanin at room temperature by the chlor-amine-T method. Galanin, 10 µg, in 20 µl 0.05 M sodium phosphate buffer (pH 7.5) and 20 µl chloramine-T (5 mg/ml) are added in a batch containing 2 mCi Na¹²⁵I (245 mCi/ml). The reaction is terminated by adding 100 µl of a solution of sodium metabisulfite (1.2 mg/ml). The reaction mixture is transferred onto a column packed with SP-Sephadex C25, equilibrated with a solution of 100 µg/ml BSA, then washed and equilibrated with 0.05 M sodium phosphate buffer (pH 5.0). The excess of Na¹²⁵I is first eluted with 0.05 M sodium phosphate buffer (pH 5.0), while the iodinated galanin is eluted as a single peak with 0.05 M sodium phosphate buffer (pH 8.1). Fractions corresponding to a peak of ¹²⁵I-galanin are pooled, the pH adjusted to 6.0 with acetic acid, and the aliquots stored at -18°C until use.

Male Sprague-Dawley rats are sacrificed and their brains quickly removed. The hypothalamus is dissected for **preparation of membranes**. The tissue is homogenized (10% mass/vol) in 0.32 M sucrose buffered with 5 mM HEPES (pH 7.4). The homogenate is diluted 10-fold and centrifuged at 1000 g for 10 min. The supernatant is centrifuged at 10,000 g for 45 min, and the pellet is resuspended in 5 mM HEPES-buffered Krebs-Ringer solution (pH 7.4).

Displacement experiments are carried out with various test compounds in a final volume of 400 µl HEPES/Krebs-Ringer solution, 0.05% BSA (pH 7.4), containing 1 mM ¹²⁵I-galanin and 70–100 µg membrane preparation. Samples are incubated for 30 min at 37°C. Incubation is terminated by the addition of 10 ml ice-cold HEPES/Krebs-Ringer solution, followed by rapid filtration over Whatman GF/B filters, precoated for 5–6 h in 0.3% polyethyleneimine solution. Specific binding is defined as that displaceable by 1 mM rat galanin1–29.

EVALUATION

Bound/total count is calculated and displacement curves versus the standard are established.

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L.4.5

Adipsin

L.4.5.1

General Considerations on Adipsin

Proteins of the alternative complement pathway are secreted by adipose tissue (Choy et al. 1992; Peake et al. 1997). **Adipsin (complement D)** was the first to be cloned from an adipose tissue cell line and shown to be synthesized and secreted by adipose tissue (Cook et al. 1987; Flier et al. 1987; Johnson et al. 1991; White et al. 1992). Adipsin is markedly suppressed in *ob/ob*, *db/db*, monosodium glutamate-induced obese mice, in obese JCR:LA-cp rats and in cafeteria-fed rats (Rosen et al. 1989; Dugail et al. 1990; Shillabeer et al. 1992; Spurlock et al. 1996), and is regulated by glucocorticoids, retinoic acid, sympathomimetic drugs and in-

sulin (Kitagawa et al. 1989; Spiegelman et al. 1989; Lowell and Flier 1990; Lowell et al. 1990; Moustaid et al. 1990; Antras et al. 1991; Miner et al. 1993). In contrast to rodents, adipsin increases with adiposity in humans and in response to feeding and is decreased during fasting, cachexia and lipatrophy (Napolitano et al. 1994).

Adipsin is required for the synthesis of **acylation stimulating protein (ASP)**, a protein implicated in fat metabolism (Sniderman and Cianflone 1994; Cianflone et al. 1999; van Harmelen et al. 1999). ASP is produced by the cleavage of C3a by carboxypeptidase and is highly expressed by mature adipocytes. The synthesis of C3a from C3 requires complement factor B and adipsin. Plasma ASP increases with meals and facilitates the synthesis and storage of triglycerides. Consistent with its role as a mediator of lipogenesis, ASP deficiency increases postprandial fatty acid levels and decreases weight gain and triglyceride synthesis in mice (Murray et al. 1999, 2000).

Several other factors, such as adiponectin (Takahashi et al. 2000) are involved in the function of adipose tissue acting as target as well as an endocrine organ (Ahima and Flier 2000).

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- 1989; Lowell et al. 1990; Dugail et al. 1990) and rats (Miner et al. 1993).

PROCEDURE

Various strains of mice (CD-1 mice, C57BL6 *ob/ob* mice, C57BL/Ks misty diabetes (m db/m db) mice, and mice with monosodium glutamate induced obesity) are treated with drugs, e. g., ephedrine (1 g/kg chow) and caffeine (1.4 g/kg chow) mixed in the diet. The effects of these regimens on fat pad weight, specific mRNAs in white epididymal and brown adipose tissue and serum adipsin concentrations are assessed.

RNA is extracted from epididymal adipose tissue, brown intercapsular adipose tissue, 3T3-F442A adipocytes and isolated rat adipocytes by the guanidium-cesium chloride technique. Total RNA is denatured, electrophoresed in 1.5% agarose, transferred to nylon filters, and hybridized to random primed adipsin, uncoupling protein (UCP), or actin cDNAs (Flier et al. 1987).

Serum adipsin and adipsin released by 3T3-F442A cultured adipocytes are assessed by RIA. Mouse adipsin purified from Chinese hamster ovary cells stably transfected with an adipsin expression vector is iodinated using the Bolton-Hunter reagent. The labeled protein is separated from unincorporated ¹²⁵I using G-50 Sephadex chromatography. The first rabbit polyclonal antibody is raised to purified mouse adipsin overexpressed using a baculovirus expression system. The assay is carried out in PBS, pH 7.4, supplemented with CaCl₂ (0.1 g/liter), MgCl₂ (0.1 g/liter), and BSA (0.1%). Serum adipsin is assessed in a dilution of 1:1000–1:5000 and in culture media at a dilution of 1:100. After adding tracer, standards, and serum, the tubes are mixed and the first antibody is added at a dilution of 1:800. After an overnight incubation, the bound and unbound tracer are separated using goat anti-rabbit immunoglobulin C fixed to heat killed staphylococcus at a ratio 1:1.2.

EVALUATION

Standard curves are generated using mouse adipsin purified from stably transfected CHO cells. Serial dilutions of standards, serum, and culture media are compared.

MODIFICATIONS OF THE METHOD

Dugail et al. (1989) showed that, in sharp contrast with genetically obese mice, adipsin mRNA is not suppressed in genetically obese Zucker rats.

Platt et al. (1994) found that a tissue-specific transcription factor that regulates adipsin expression is less active in the adipose tissue of obese animals.

L.4.5.2

Adipsin Expression in Mice

PURPOSE AND RATIONALE

Adipsin expression at the protein and mRNA levels was studied in mice (Flier et al. 1987; Spiegelman et al.

Napolitano et al. (1994) determined concentrations in blood and rates of adipin secretion by adipose tissue in humans using a two-monoclonal 'sandwich' ELISA assay.

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L.4.6 Ghrelin

Small synthetic molecules called growth hormone secretagogues (GHSs) (Bowers et al. 1980, 1984) stimulate the release of growth hormone (GH) from the pituitary. They act through the GHS-R, a G-protein-coupled receptor (Howard et al. 1996). The natural ligand of this receptor was discovered as a 28-amino-acid-containing peptide, called ghrelin (Kojima et al. 1999, 2000, 2001; Davenport et al. 2005), produced in the XA-like cells of the stomach (Kojima et al. 1999; Date et al. 2000); however, smaller amounts of ghrelin are also found in the small and large intestine (Hosoda et al. 2000). Ghrelin and the growth hormone secretagogue receptor are expressed in the rat adrenal cortex (Andreis et al. 2003). GH, GH receptor, GH secretagogue receptor, and ghrelin are also expressed in human T cells, B cells, and neutrophils (Hattori et al. 2001).

Ghrelin is the first peptide hormone in which the third amino acid serine is modified by a fatty acid; this modification is essential for the peptide's biological activity. Beside ghrelin's GH-releasing effect, it is a powerful appetite-stimulating peptide. Plasma ghrelin concentration is increased in fasting conditions and reduced after habitual feeding (Kojima and Kangawa 2005), suggesting that ghrelin may act as an initiation signal for food intake. Chronic systemic or intracerebroventricular application to rats produced hyperphagia and obesity in rats (Wren et al. 2001). The appetite-stimulatory signal of ghrelin is mediated through action on the hypothalamic neuropeptide Y (NPY) and the Y1 receptor (Asakawa et al. 2001).

[¹²⁵I-His⁹]-ghrelin was recommended as a radioligand for localizing GHS and GH receptors in human and rat tissue (Katugampola et al. 2001).

Various heterocyclic compounds acting as ghrelin receptor agonists have been developed, which might be useful for the treatment of anorexia nervosa (Palucki et al. 2001). Multiple ghrelin-derived molecules produced by post-translational processing were identified indicating structural divergence of human ghrelin (Hosoda et al. 2003). Until now, however, no specific ghrelin receptor antagonist has been described, although such a compound might be an attractive approach to reduce feed intake and to decrease obesity.

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L.5 Safety Pharmacology of Drugs with Anti-obesity Activity

See Herling (2006).

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Chapter M

Anti-Atherosclerotic Activity¹

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¹Reviewed and amended by H.-L. Schäfer

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M.1 Induction of Experimental Atherosclerosis

M.1.0.1 General Considerations

Experimental atherosclerosis was first successfully induced in rabbits by Saltykow (1908) and Ignatowski (1909). During the following years, various scientists found that dietary cholesterol was the responsible stimulus for development of atherosclerosis. Other species are also susceptible to diet-induced atherosclerosis (Reviews by Kritchevsky 1964; Hadjiinky et al. 1991). Mouse models of atherosclerosis were reviewed by Reardon and Getz (2001) and by Daugherty (2002). Tailleux et al. (2003) underlined the importance of nuclear receptors, e. g., peroxisome proliferators-activated receptors (PPARs), liver X receptors (LXR) (Joseph and Totonoz 2003; Terasaka et al. 2003) and retinoid receptors (RXR) (Staels 2001; Li et al. 2005), which are implicated in lipid metabolism and inflammation.

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M.1.0.2 Cholesterol-Diet-Induced Atherosclerosis in Rabbits and Other Species

PURPOSE AND RATIONALE

Rabbits are known to be susceptible to hypercholesterolemia and arteriosclerosis after excessive cholesterol feeding. Therefore, this approach has been chosen by many authors to study the effect of potential anti-arteriosclerotic drugs.

PROCEDURE

Several modifications of the protocol have been described. Usually, male rabbits from an inbred strain, e. g., white New Zealand, at an age of 8–10 weeks are used. Body weight variation should be as low as possible. At the beginning of the experiment, blood is withdrawn from the marginal ear vein for determination of total cholesterol, total glycerides, and blood sugar. Groups of 10 animals are used for treatment with drugs or as controls. The rabbits are switched from commercial food to a diet supplemented with 0.3–2% cholesterol and kept on this regimen for a period of 10–12 weeks. One group is kept on normal diet. During and at the end of the experiment blood is taken for analysis. Usually, cholesterol and triglyceride levels increase several-fold over the original values.

The animals are sacrificed and the thoracic aorta is removed, cleaned of surrounding tissues, and longitudinally cut and opened for fixation with formaldehyde. The tissue is stained with oil red. The percentage of

the intimal surface covered by the oil red positive lesions is calculated with a computerized planimeter. In animals fed a normal diet, the aorta does not show any staining, whereas in cholesterol-fed rabbits the aorta shows severe atherogenic lesions.

EVALUATION

Data are expressed as means \pm standard deviation. Statistical evaluation is performed by Dunnett's or Scheffé's test. A *p*-value of <0.05 is regarded as statistically significant.

MODIFICATIONS OF THE METHOD

Shore and Shore (1976) studied two **different strains of rabbits** (New Zealand White and Dutch Belt) as models of hyperlipoproteinemia and atherosclerosis.

Tao et al. (2003) studied antioxidative, antinitrative, and vasculoprotective effects of a PPAR- γ agonist in New Zealand White rabbits with hypercholesterolemia induced by a high-cholesterol diet.

Studies of Kritchevsky et al. (1989) on experimental atherosclerosis in **rabbits fed cholesterol-free diets** revealed a greater influence of animal protein and of partially hydrogenated soybean oil on development of atherosclerosis than plant protein and unsaturated soybean oil.

Cockerels (Tennent et al. 1960) and **turkeys** (Simpson and Harms 1969) are very susceptible to cholesterol feeding and develop marked hypercholesterolemia in rather short periods. Atherosclerosis could also be induced in cockerels by high doses of estrogen without atherogenic diet (Caldwell and Suydam 1959).

Spontaneous arteriosclerosis in **pigeons** has been described by Clarkson and Lofland (1961).

The **Japanese sea quail** (*Coturnix coturnix japonica*) is highly susceptible to the rapid development of severe experimental atherosclerosis (Day et al. 1975, 1977, 1979, 1990; Chapman et al. 1976).

Out of 13 strains of **mice**, Roberts and Thompson (1976) selected the C57BR/cdJ and the CBA/J strain and used these strains and their hybrids as models for atherosclerosis research.

Paigen et al. (1987) described quantitative assessment of atherosclerotic lesions in mice. After 14 weeks on an atherogenic diet C57BL/6J female mice had aortic lesions at each of the coronary arteries, at the junction of the aorta to the heart and in scattered areas of the aortic surface. The lesions increased after 9 months of atherogenic diet. Methods of evaluating the number and size of lesions were compared including sizing with a microscope eyepiece grid and computer-assisted planimetry.

Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice were described by Paigen et al. (1990).

Yamaguchi et al. (1993) found that addition of 10% linoleic acid to a high-cholesterol diet enhanced cholesterol deposition in the aorta of male ICR strain mice.

In **rats** hypercholesterolemia can be induced by daily administration by gavage of 1 ml/100 g body weight of a cocktail containing in 1 l peanut oil: 100 g cholesterol, 30 g propylthio-uracil, and 100 g cholic acid over a period of 7 days. The test compounds are administered simultaneously with the cocktail (Fillios et al. 1956; Lustalot et al. 1961).

Inoue et al. (1990) induced experimental atherosclerosis in the rat carotid artery by balloon de-endothelialization and atherogenic diet. A balloon catheter was introduced into the rat's carotid arteries from the iliac arteries and the endothelium was denuded.

The **hamster** is susceptible to atherosclerosis. Nistor et al. (1987) fed male hamsters a hyperlipidemic diet consisting of standard chow supplemented with 3% cholesterol and 15% commercial butter for 12 months. Serum total cholesterol doubled after 3 weeks and attained a 17-fold value after 10 months. Up to 6 months, smooth muscle cells in the intima and media of the aorta as well as endothelial cells began to load with lipids. After 10 months the affected zones looked like human atherosclerotic plaque with huge cholesterol crystal deposits, calcium deposits and necrosis.

Especially the hybrid hamster strain Bioä F₁B (Bio Breeders Fitchburg, MA, USA) is more susceptible to dietary induced atherosclerosis than other strains (Kowala et al. 1991). Early atherosclerotic lesions can be induced within a 3-months-feeding of a cholesterol/butter enriched diet. In these animals simvastatin dose-dependently inhibited the development of hyperlipidemia and the plaque formation by cholesterol synthesis inhibition. The histopathological examination of the aortas showed that the cholesterol/butter fed F₁B hamster developed atherosclerotic lesions and functional changes in the aorta which are closely related to man (Schäfer et al. 1999).

Soret et al. (1976) studied the diet-induced hypercholesterolemia in the diabetic and non-diabetic Chinese hamster.

Beitz and Mest (1991) used cholesterol-fed **guinea pigs** to study the antihyperlipemic effects of a potentially anti-atherosclerotic drug. Fernandez (2001) reviewed the advantages of the guinea pig as an animal model to study hepatic cholesterol, lipoprotein metabolism, and early atherosclerosis. Guinea pigs de-

velop fatty streaks after 12 weeks on a cholesterol diet; the response to cholesterol is highly individualized, and animals can be a hyper-responder or a hyporesponder to dietary cholesterol.

During the last 30 years **minipigs** have become a popular animal model for testing lipid-lowering drugs and their anti-atherosclerotic properties (Jacobsson 1987; Huff et al. 2002; Telford et al. 2003). Jacobsson and Lundholm investigated the lipid-lowering and anti-atherosclerotic effects of clofibrate, nicotinic acid, and other drugs (Lundholm 1978; Jacobsson and Lundholm 1982; Jacobsson 1987). Huff and Telford investigated the effect of atorvastatin and a bile acid absorption inhibitor on lipid metabolism and serum LDL kinetics (Huff et al. 2002; Telford et al. 2003). The benefits of the minipig are the absence of rodent-specific peroxisomal proliferation by PPAR α -agonists and closer homology to humans in absolute and relative organ sizes. The effective doses of the tested drugs in the minipig were close to clinically effective doses in humans.

Malinow et al. (1976) recommended the **cynomolgus monkey** as a model for therapeutic intervention on established coronary atherosclerosis.

This species was used by Hollander et al. (1978) to study the development atherosclerosis after a cholesterol and fat enriched diet.

Beere et al. (1992) described experimental atherosclerosis at the carotid bifurcation of the cynomolgus monkey by a cholesterol-enriched diet.

Eggen et al. (1991) studied the progression and the regression of diet-induced atherosclerotic lesions in aorta and coronary arteries on **rhesus monkeys**.

Howard (1976) recommended the **baboon** as model in atherosclerosis research because of the similarity of cholesterol metabolism and composition of the lipoproteins to man.

Kushwaha et al. (1991) determined the effect of estrogen and progesterone on plasma cholesterol concentrations and on arterial lesions in ovariectomized and hysterectomized baboons fed a high-cholesterol/high-saturated-fat diet.

Blaton and Peeters (1976) reported studies on the **chimpanzee**, the **baboon** and the **rhesus macacus** as models for atherosclerosis.

The use of normal adult **marmosets**, a species with a lipoprotein profile similar to that of humans, may be an alternative (Crook et al. 1990; Baxter et al. 1992).

Ming-Peng et al. (1990) studied high density lipoproteins and prevention of experimental atherosclerosis in **tree shrews** (*Tupaia belangeri yunalis*). In contrast to rabbits, no increased lipid deposition in aor-

tic intima after cholesterol feeding was found in tree shrews.

CRITICAL ASSESSMENT OF THE METHOD

Diet-induced hypercholesterolemia is useful for the detection of agents that interfere with the adsorption, degradation, serum clearance, and excretion of cholesterol to receive a broader effect range. It must be considered whether the effects of agents that interfere with cholesterol absorption will be enforced and therefore whether the efficacy is overestimated in those models.

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M.1.0.3

Hereditary Hypercholesterolemia in Rats

A strain of genetically hypercholesterolemic rats (RICO) was described by Müller et al. (1979). In contrast to Zucker-rats, these animals are normo-triglyceridemic and non-obese. The hypercholesterolemia of the RICO rat is related to a decreased rate of catabolism of chylomicrons and LDL, but more specifically to an excessive production of these two types of lipoproteins. This strain has been proposed to study hypolipidemic drugs, particularly those designed to decrease the plasma concentrations of chylomicrons and LDL. Hypolipidemic effects of β -cyclodextrin were found in this model (Riottot et al. 1993).

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M.1.0.4

Hereditary Hyperlipemia in Rabbits

Watanabe et al. (1977, 1980) described a strain of rabbits with hereditary hyperlipemia (WHHL rabbit) which has been used by several scientists to study development of atherosclerosis, as well as for histological and functional changes of the aorta. At the age of 10–14 months homozygous animals exhibit an atheromatous plaque, distributed heterogeneously over the luminal surface of the aorta. Serum cholesterol is increased up to 400–600 mg/dl. The increased levels of LDL have been studied in detail (Kita et al. 1981, 1982).

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M.1.0.5 Studies in Transgenic Mice

Mice do not develop atherosclerotic lesions spontaneously. Paigen et al. (1985) fed ten inbred strains of mice an atherogenic diet containing 1.25% cholesterol, 0.5% cholic acid and 15% fat in order to study the variation in susceptibility to atherosclerosis among these strains. The strains were examined for plasma cholesterol and triglyceride levels and for formation of lipid-containing lesions in the aortic wall. The most susceptible strain was C57BL/6J mice.

Several transgenic animals as disease model were created during the last decade, mostly mice (Stoltzfus and Rubin 1993), but also rats and rabbits.

The widely used model is the Apo E knockout mouse originally created by Nubuyo Maeda, University of North Carolina, Chapel Hill, NC. These Apo E knockout mice have spontaneously elevated plasma cholesterol levels, and develop atherosclerosis even on regular chow within 3–4 months. The time dependent progression of atherosclerosis leads to lesions similar in histopathology to those observed in humans. This animal model is used as background for atherosclerosis research and target validation. Duez et al. (2002) used apolipoprotein E-deficient mice and human apoA-I transgenic apoE-deficient mice fed a Western diet to investigate the reduction of atherosclerosis by the PPAR α agonist fenofibrate.

Walsh et al. (1989) and Rubin et al. (1991) integrated human apolipoprotein A-I gene in transgenic mice resulting in an increase of HDL levels.

Linton et al. (1993) described the development of transgenic mice expressing high levels of human apolipoprotein B48 and human apolipoprotein B100 which are considered to be atherogenic.

Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet (Purcell-Huynh et al. 1995).

Ishibashi et al. (1994) found massive xanthomatosis of the skin and subcutaneous tissue and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice.

Sasan et al. (1998) reported that low density lipoprotein receptor-negative mice expressing human apolipoprotein B-100 develop complex atherosclerotic lesions on a chow diet.

Li et al. (2000) used LDL receptor-deficient mice to study the inhibition of development of atherosclerosis by PPAR γ -specific agonists.

Joseph et al. (2002) investigated the inhibition of development of atherosclerosis in *LDL receptor* knockout mice and *ApoE* knockout mice by a synthetic LXR ligand.

Terasaka et al. (2003) reported inhibition of development of atherosclerosis in LDL-receptor-deficient mice by a synthetic liver X receptor ligand.

Transgenic mice lacking apolipoprotein E showed severe hypercholesterolemia and atherosclerosis (Plump et al. 1992; Zhang et al. 1992). ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree (Nakashima et al. 1994).

Fu et al. (2003) reported that the PPAR α agonist ciprofibrate severely aggravates hypercholesterolemia and accelerates the development of atherosclerosis in mice lacking apolipoprotein E.

Wang (2005) described the cardiovascular functional phenotype of ApoE knockout mice and reported that simvastatin paradoxically increased serum lipids and atherosclerosis, but decreased serum lipids and atherosclerosis in LDL receptor knockout mice.

Meier and Leitersdorf (2004) reviewed nutritional, pharmacologic, and genetic atherosclerosis intervention studies in ApoE knockout mice. Several known beneficial nutritional interventions are effective, as well as ACE inhibitors, Ang II receptor blockers, the cholesterol absorption inhibitor ezetimibe and PPAR γ agonists. Meier pointed out that the model helps the understanding that atherosclerosis is a form of chronic inflammation and that, despite being imperfect, the ApoE knockout mouse is currently the most popular mouse model used by researchers around the globe.

Overexpression of apolipoprotein E in transgenic mice reduced plasma cholesterol and triglyceride levels, prevented hypercholesterolemia and inhibited the formation of fatty streak lesions (Harada et al. 1996).

Transgenic mice overexpressing the human dysfunctional apolipoprotein E variant APOE*3 Leiden develop hyperlipidemia and are highly susceptible to diet-induced atherosclerosis (Groot et al. 1996).

Sullivan et al. (1998) reported type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse ApoE with human ApoE*2.

Severe hypercholesterolemia, hypertriglyceridemia, and atherosclerosis were observed in ob/ob mice lacking both leptin and the LDL receptor (Hasty et al. 2001).

Based on animal knockout, transgenic, and gene therapy studies, Bursill et al. (2004) highlighted the importance of chemokines, particularly the CC-chemokines and fractalkine, in atherosclerosis.

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M.1.0.6**Evaluation of Endothelial Function in Rabbits with Atherosclerosis****PURPOSE AND RATIONALE**

Cholesterol feeding of rabbits impairs the endothelium-dependent relaxation evoked by acetylcholine in the aorta. This phenomenon can be used to study the influence of vasodilators as well as the prevention by ACE-inhibitors (Becker et al. 1991).

PROCEDURE

Male white New Zealand rabbits weighing 3–4 kg receive a hypercholesterolemic diet containing 0.25 to 1% cholesterol and 3% coconut oil. Rabbits of the same weight receiving standard diet serve as controls. After several weeks, the serum cholesterol levels are increased from 30–40 mg/dl in the control group up to 900–1000 mg/dl in the cholesterol-fed group. At the end of the treatment period, the animals are sacrificed by intravenous injection of sodium pentobarbital and a complete autopsy is performed.

Intact proximal parts of the thoracic aorta are sectioned into 2 mm wide rings, cut off to strips,

and suspended at 2 g gauge in 25 ml organ chambers filled with a buffer solution of 37°C comprising 113.8 mM NaCl, 20 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.1 mM MgSO₄, 2.5 mM CaCl₂, and 5.5 mM glucose being gassed with a 95% O₂/5% CO₂ mixture to achieve a pH of 7.4. After 2 h, when a stable contractile tone is established, norepinephrine is added at a final concentration of 1×10^{-8} M, which produces a stable submaximum isotonic contraction. Then, acetylcholine is added in 10 fold incremental doses from 1×10^{-8} M up to final concentrations of 10^{-5} M. Relaxation of aortic strips is assessed as percentage decrease in contraction. Acetylcholine induced concentration-dependent relaxation is greatly impaired in aorta rings from cholesterol-fed rabbits whereas contractions to norepinephrine are only slightly diminished.

EVALUATION

The data are expressed as mean \pm SEM and compared by Student's *t*-test for unpaired data.

MODIFICATIONS OF THE METHOD

Verbeuren et al. (1986, 1990) and Tagawa et al. (1991) used a bioassay for the determination of EDRF (Rubanyi et al. 1985). The donor thoracic aorta segments (3 cm long) are mounted horizontally in perfusion chambers filled with physiological salt solution which contains indomethacin (3×10^{-6} M) at 37°C; the solution is gassed with a mixture of 20% O₂, 5% CO₂, and 75% N₂. The aortas (control and atherosclerotic tissue) and a piece of glass tubing are perfused continuously with the same solution at 3 ml/min. The perfusate is dripping directly onto a segment (5 mm long) of either a control or an atherosclerotic abdominal aorta from which the endothelium was mechanically removed. The detector abdominal aortas are mounted vertically over two hooks and the development of isometric tension is monitored continuously. The initial tension of the detector aortas is set at 8 g. The perfusate of each donor aorta is analyzed both on a control and on an atherosclerotic detector preparation: the order of this double analysis is selected at random. A control and an atherosclerotic donor aorta are always analyzed in parallel and thus on the same detector tissue. Drugs are added to the perfusion medium. Before the start of the experiments, the tissues are allowed to equilibrate for 45 min in the perfusion chambers. By means of a three-way system, placed at the outlet of the donor aortas, atropine (10^{-6} M) is added to the perfusate to block any muscarinic contractile effect of acetylcholine on the detector tissue. Nora-

drenaline (2×10^{-6} M) is infused into the perfusate via this 3-way system causing a sustained contraction in the detector tissue.

After the stabilization of the contraction to noradrenaline, the highest dose of acetylcholine (1.6×10^{-8} mol) is added to the solution for perfusion of the tubing in order to confirm no change in the contractile response to noradrenaline in the detector tissues. Moreover, the perfusate from the control and atherosclerotic donor aortas under basal conditions does not significantly affect the contraction of the detector tissues. The tissues are allowed to equilibrate and then increasing doses of acetylcholine (0.01 to 160×10^{-10} mol) are injected into the perfusion medium close to the donor aortas. Relaxation is less pronounced in the detector tissues when atherosclerotic aortas are stimulated with acetylcholine indicating that the cholinergic agent causes a smaller release of EDRF from atherosclerotic donor aortas than from control donor aortas. The degree of the arteriosclerotic lesion can be assessed by this functional assay.

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M.1.0.7**Intimal Reactions After Endothelial Injury****PURPOSE AND RATIONALE**

Several attempts have been made to induce intimal injury in animals which is followed by proliferation and formation of fatty streaks similar to the alterations found in human atherosclerosis. One approach is the "balloon catheterization".

PROCEDURE

Male New Zealand White rabbits weighing 2.0–3.5 kg or male Sprague-Dawley rats weighing 350–400 g are used. An embolectomy catheter (Edwards Laboratories, size 4 French for rabbits, size 2 French for rats) is introduced into the right femoral artery under surgical anesthesia and passed to the aortic arch. After inflation with room air, the catheter is withdrawn to the iliac bifurcation, deflated and removed.

The incorporation of [³H]thymidine into DNA of rabbit or rat aorta is measured 48 h after balloon catheterization. Animals are sacrificed 45 min after intravenous injection of [³H]thymidine. Intima-media is prepared from whole aorta by scraping with blunt forceps. The specific activity of ³H in DNA is determined after extraction of the DNA in the washed tissue homogenate using hot dilute perchloric acid. DNA is assayed by the diphenylamine method. ³H Incorporation is measured in a liquid scintillation counter.

For histological examination, rabbits are injected 2 weeks after balloon catheterization intravenously with heparin (500 U/kg) and then sacrificed. Fixative (2% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4) is infused at a constant pressure of 100 mm Hg via a carotid cannula and the aorta is pressure fixed *in situ* for 60 min. Intimal proliferation is quantified in the upper abdominal aorta, in the lower abdominal aorta and half-way between these points.

EVALUATION

[³H]Thymidine incorporation and intimal proliferation is compared between drug treated animals and controls.

MODIFICATIONS OF THE METHOD

DeCampi et al. (1988) studied the effects of various drugs on accelerated myointimal proliferation in canine veno-arterial allografts by histological methods. An 8-cm length of femoral vein was removed, reversed, divided, and sewn end-to-end into carotid or femoral arteries of a recipient dog.

Berkenboom et al. (1989) induced experimental

atherosclerosis in **canine** and **porcine** coronary arteries by endothelial denudation followed by a high-cholesterol diet.

Kawata et al. (1990) described the detection of regenerating cells in the aorta after ballooning by immunocytochemical demonstration of the thymidine analogue 5-bromo-2'-deoxyuridine.

Manderson et al. (1990) described changes in vascular reactivity of carotid arteries in rabbits following endothelial denudation.

Bocan et al. (1991) tested an ACAT inhibitor and selected lipid-lowering agents for anti-atherosclerotic activity in iliac-femoral and thoracic aortic lesions. Atherosclerotic lesion comparable in composition to human fatty streaks were induced by chronic endothelial denudation in the iliac-femoral artery inserting a sterile, indwelling, 18-cm nylon filament with a diameter of 200 μm into the lumen of the right femoral artery in hypercholesterolemic New Zealand White rabbits. Naturally occurring fatty streaks developed in the thoracic aorta following cholesterol feeding. The effect of treatment on lesion regression was evaluated in the iliac-femoral artery, while changes in the lesion progression were evaluated in the thoracic artery.

Davies et al. (1993) performed right common carotid artery bypass grafting using the ipsilateral external jugal vein in New Zealand White rabbits and studied the response to endothelin-1 in normolipidemic and hyperlipidemic animals.

Groves et al. (1993) studied platelet adhesion and thrombus formation in a **porcine** model of balloon angioplasty.

ACE inhibitor treatment reduced the neointima formation after endothelial denudation in the carotid artery of **rats** using a balloon catheter (Farhy et al. 1992; Linz and Schölkens 1992; Linz et al. 1993, 1994).

Lyle et al. (1995) tested the effect of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition in an atherosclerotic rabbit model of angioplastic injury. Acute ¹¹¹indium-labelled platelet deposition and thrombosis were assessed 4 h after balloon-injury in femoral arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month).

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M.2 Influence on Lipid Metabolism

M.2.0.1

General Considerations

Elevated lipid levels, especially hypercholesterolemia, result from increased absorption from the gut, enhanced endogenous synthesis, or inadequate clearance from serum. Therefore, there are three feasible ways to reduce hyperlipidemia: to block endogenous synthesis, decrease absorption or enhance clearance from

serum. These three factors can be evaluated in normal animals without artificial diets. Clinically used lipid-lowering compounds such as PPAR α agonists (fibrates), cholesterol absorption inhibitors (ezetimibe), bile acid sequestrants, and HMG-CoA reductase inhibitors (statins) can be tested in this way, and their pharmacological activity further investigated with additional tests. For the investigation of the effects on plasma lipids, the right animal model has to be chosen. For fibrates, rats and mice are appropriate models; for LDL-lowering compounds, hamster, guinea and rabbits. Earlier attempts to interfere with endogenous cholesterol synthesis resulted in the accumulation of sterols other than cholesterol (Holmes 1964). To date only the inhibition of cholesterol biosynthesis with HMG-CoA reductase inhibitors has been a clinically effective approach for LDL cholesterol lowering. Inhibition of other enzymes of the cholesterol biosynthesis pathway upstream from HMG-CoA reductase, such as squalene synthetase, were investigated by several pharmaceutical companies until the 1990s, but this development was discontinued for safety reasons. Inhibition of cholesterol biosynthesis upstream from HMG-CoA reductase leads to non-physiological accumulation of metabolic intermediates. In the past the inhibition of cholesterol absorption by ACAT-inhibitors was a widely followed approach. ACAT-inhibitors inhibit cholesterol absorption in rodents effectively, but so far all ACAT-inhibitors have been ineffective in humans. The only compound that is effective in humans is ezetimibe, an azetidinone. The compound was discovered fortuitously in an ACAT-inhibitor program. During the development of that compound, it was found that ezetimibe inhibits cholesterol absorption independently from ACAT (Van Heek et al. 2000, 2001, 2003; Harris et al. 2003; Clader 2004). Ezetimibe inhibits cholesterol absorption in several animal models and is effective against plasma LDL-cholesterol in humans. Another approach to enhance hepatic LDL clearance is to interrupt bile acid recirculation. Compounds that inhibit bile acid absorption increase the conversion of cholesterol into bile acids, enhance hepatic clearance, and lower LDL cholesterol by LDL receptor upregulation in the liver.

Protective effects of calcium antagonists against experimental arteriosclerosis acting mainly on other mechanisms than lipid metabolism have been claimed by various authors but the clinical relevance is still questionable (Kjeldsen and Stender 1989; Fronck 1990; Knorr and Kazda 1990; Fleckenstein-Grün et al. 1992).

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M.2.0.2**Hypolipidemic Activity in Rats****PURPOSE AND RATIONALE**

Hyperlipoproteinemia with increased concentrations of cholesterol- and triglyceride-carrying lipoproteins is considered to be the cause of arteriosclerosis with its dual sequelae of thrombosis and infarction. Lipoproteins are divided into 6 major classes: chylomicrons, chylomicron remnants, VLDL (very low density lipoproteins), IDL (intermediate density lipoproteins), LDL (low density lipoproteins), and HDL (high density lipoproteins). HDL promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver. Therefore, increased levels of HDL are desirable. On the contrary, high levels of VLDL and LDL promote arteriosclerosis. LDL, especially in its oxidized form, is taken up by macrophages via a scavenger mechanism. Therefore, anti-arteriosclerotic drugs should reduce VLDL and LDL and/or elevate HDL.

PROCEDURE

Groups of ten male Wistar or Sprague Dawley rats weighing 180–200 g are used. They are given once daily in the morning over a period of 5–8 days the test compounds or the standard in various doses ranging from 1 to 100 mg/kg via a stomach tube in a volume of 5 ml/kg. The control group is given the solvent (e. g., 0.5% HEC with 0.4% Tween 80) only. The body weight of each animal is recorded at the beginning and at the end of the experiment. If the effect on VLDL and triglycerides is the focus of the experiment, animals should be non-fasted or postprandial, and food should be withdrawn for no longer than 4 h before blood sampling. On the morning of the first day, blood samples are taken under light isoflurane or CO₂ anesthesia by retro-orbital puncture. Then, the first dose is applied. During the whole period, the animals have free access to food and water. At the end of the experiment, blood samples are taken by retro-orbital puncture. Immediately thereafter, the animals are sacrificed and the liver removed, blotted free from blood and weighed. Samples of liver are frozen in liquid nitrogen and stored at –25°C for lipid analysis. The blood samples are centrifuged for 2 min at 16,000 g. Total cholesterol and triglycerides in each blood sample are determined.

To estimate the serum lipoproteins two methods are available: ultracentrifugation and liquid chromatography. For ultracentrifugation more than 1 ml serum is needed, so the serum has to be group-vice pooled. The serum lipoproteins are separated by means of a preparative ultracentrifuge.

The separation of fractions VLDL, LDL, HDL, and of the subnatant of HDL is carried out as follows:

| | |
|------------------|-------------------------------------------------------------|
| VLDL | native density of the serum (1.006), 16 h at 40,000 rpm, |
| LDL | density range from 1.006 to 1.04, 18 h at 40,000 rpm, |
| HDL | density range from 1.04 to 1.21, 18 h at 40,000 rpm, |
| Subnatant of HDL | density > 1.21 |

The density is adjusted by addition of a calculated amount of NaBr solution.

Another faster approach is the separation of lipoprotein by fast protein liquid chromatography (FPLC).

Distribution of cholesterol to lipoprotein fractions is performed after separation of fractions by gel permeation chromatography, using a published method (März et al. 1993). The cholesterol and triglyceride content in the isolated lipoprotein fractions after ultracentrifugation or FPLC separation is determined using standard enzymatic assays for clinical diagnostics

(e. g., Roche-Diagnostics, CHOD-PAP method, Siedel et al. 1983). Triglycerides are also determined with standard enzymatic assays for clinical diagnostic (e. g., Roche Diagnostics, GPO-PAP method; Eggstein and Kreutz 1966a, 1966b; Wahlefeld 1974). The method of Lowry et al. (1951) is applied to determine protein content.

Frozen samples of liver are thawed, homogenized and aliquots are extracted with chloroform/methanol or dichloromethane/methanol 2:1 (v/v). The extracts are purified according to Folch et al. (1957). Solvents of aliquots of the extracts are evaporated and dissolved in isopropanol for determinations of cholesterol and triglycerides, using enzymatic assays for serum diagnostics (Herling et al. 1999).

EVALUATION

Average values of body weight, cholesterol and triglycerides are expressed as percentage of initial values for each group at the end of the experiment. Statistical differences between the controls and the treatment groups are evaluated by covariance analysis.

CRITICAL ASSESSMENT OF THE METHOD

Studies with normocholesterinemic animals are faced with the difficulty that starting cholesterol levels are relatively low, and to achieve significance for lowering of cholesterol levels requires large groups of animals. LDL cholesterol lowering can be investigated in normocholesterolemic hamster, guinea pigs or minipigs, not in mice or rats. Triglyceride-lowering drugs such as fibrates are very effective against serum triglycerides and cholesterol in normolipemic male Sprague Dawley rats.

MODIFICATIONS OF THE METHOD

Similar tests can be performed in various species, e. g.:

- male NMRI mice, weighing 25–30 g, male or female C57/bl6 mice weighing 18–24 g,
- male New Zealand obese (NZO) mice, weighing 35–40 g,
- male Syrian golden hamsters, weighing 80–120 g,
- male Pirbright guinea pigs, weighing 200–250 g,
- male miniature pigs, weighing 14–22 kg.

To study long term effects, the experiments are extended for 4 weeks or 3 months.

Schurr et al. (1976) proposed high volume screening procedures for hypobetalipoproteinemic activity in rats.

März et al. (1993) described fast lipoprotein chromatography as a new method of analysis for plasma lipoproteins.

Adipokinetic actions of several hormones in slices of perirenal adipose tissue of various species were determined by Rudman et al. (1963).

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M.2.0.3

Hypolipidemic Activity in Syrian Hamsters

PURPOSE AND RATIONALE

The Syrian hamster (*Mesocricetus auratus*) is a widely used animal to study the effects of drugs and diet on lipoprotein metabolism. Several in human approved lipid lowering drug like HMG-Co A reductase inhibitors, cholestyramine or ezetimibe lower plasma cholesterol in hamster. The lipoprotein and bile acid metabolism of the hamster is closer to human than the lipoprotein and bile acid metabolism of rats and mice (Bravo et al. 1994; Suckling et al. 1991; Kris-Etheron and Dietsch 1997). The hamster has in contrast to pig, rat and mouse CETP (Cholesteryl Ester Transfer Protein) activity, similarly as seen in humans (Ha and Barter 1982, 1986; Ahn et al. 1994).

Increase in plasma cholesterol can be easily induced by adding small, physiological amounts of cholesterol to the diet (0.05–0.01 weight%). Additional saturated fat like coconut oil (5–10 weight%) has synergistic effects for induction of hyperlipidemia. (Kowala et al. 1991) A stable hyperlipidemia with a human

like lipoprotein pattern can be induced in hamster within 2–3 weeks by adding 10 weight% coconut butter and 0.2% cholesterol into the diet. Hamster HDL cholesterol can be easily measured after precipitation of VLDL+LDL cholesterol with phosphotungstic acid/MgCl₂ (Weingand and Daggy 1990, 1991). LDL and HDL cholesterol can also be measured directly with Kits from Roche Diagnostics. A complete separation of all lipoprotein fractions can be done by FPLC on Superose 6 columns (Pharmacia) with a modified method of März et al. (1993).

PROCEDURE

Male Syrian hamsters weighing 95–125 g at the start of the experiment are randomly assigned to form groups of 6–15 animals each. Test compounds can be administered by oral gavage, once or twice daily or mixed into the diet. If compounds are administered with diet, a different diet has to be prepared for each group. One control receives powdered or pellet standard hamster chow only; a second control and the treated groups, a cholesterol-enriched diet. If compounds are administered with diet a daily food consumption of 8 g should be taken in account for dose calculation. Cholestyramine, for example, is active when mixed into the diet in a concentration range between 0.3% to 2%. After 1–3 weeks on these diets, the animals are anesthetized with isoflurane, a blood sample is taken from the retro-orbital vein plexus, the superior vena cava or the abdominal aorta, and the liver is removed and weighed. Microsomes are prepared by ultracentrifugation from the livers.

Preparation of Plasma Lipoprotein Profiles

The blood is centrifuged at 6000 g for 5 min and the plasma removed. The plasma is analyzed for total cholesterol and triglycerides using colorimetric enzymatic assays for clinical diagnostics, as described in the previous chapter for rats (e. g., Roche Diagnostics). The cholesterol content of HDL and LDL was determined using enzymatic assays for clinical diagnostics (Roche Diagnostics, HDL-C plus and LDL-C plus).

Quantification of High-Density-Lipoprotein Cholesterol by Differential Precipitation

Weingand and Daggy (1990) compared the validity of differential precipitation with Mg²⁺-phosphotungstate for quantification of plasma high-density-lipoprotein cholesterol with ultracentrifugal flotation.

As precipitating agent, 0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium chloride is added. Cholesterol is determined spectrophotometrically with an

enzymatic cholesterol reagent containing microbial cholesterol esterase, in a Hitachi 705 clinical chemistry analyzer.

Fast Lipoprotein Chromatography

März et al. (1989, 1993) developed fast lipoprotein chromatography for rapid and quantitative analysis of lipoprotein fractions.

Fast lipoprotein chromatography is carried out with a chromatography system from Kontron consisting of two Model 420 pumps, a Model 432 variable-wavelength detector, and a Model 450 data system. Without using a pretreatment, 20 µl of plasma are applied to a 300-mm Superose column 6 equilibrated with 100 mmol/l Na₂HPO₄, pH 7.4, and 200 mmol/l NaCl. Lipoproteins are detected on-line at 500 nm after post-column derivatization with CHOD-PAP cholesterol reagent (Roche Diagnostics, Mannheim, Germany). The column eluate and the cholesterol reagent are mixed in a motor-driven microchamber attached to the column outlet, and the mixture is then passed through a "knitted" capillary (20–22). The flow rate of the reagent is 70 µl/min; the run time is 80 min. Under these conditions, it takes the column eluate 2 min to pass the derivatization capillary. VLDL-C, LDL-C and HDL-C are calculated on the basis of relative peak areas and total cholesterol, previously measured with an enzymatic assay in serum as described above.

Tissue Preparation

The liver is homogenized in a KCl (0.104 M) solution containing NaF (50 mM) using a glass Teflon homogenizer. The homogenate is centrifuged at 30,000 g, 5°C for 25 min. The supernatant is then centrifuged at 100,000 g, 5°C for 60 min to pellet the microsomes, which are resuspended in potassium phosphate buffer (50 mM) containing NaF (50 mM) adjusted to pH 7.4.

Determination of Enzyme Activities in Microsomal Fractions

Protein for all the following assays is determined using the method described by Bradford (1976).

HMG-CoA reductase activity is determined by quantifying the conversion of [¹⁴C]HMG-CoA to [¹⁴C]mevalonic acid lactone, based on the method described by Ingebritsen and Gibson (1981). ACAT activity in liver microsomes and intestinal cell homogenates is determined by the method described by Suckling et al. (1982), measuring the incorporation of [¹⁴C]oleoyl-CoA into cholesteryl oleate.

The cholesterol 7 α -hydroxylase activity in the microsomes is quantified by determination of the percentage conversion of [¹⁴C]cholesterol to 7 α -hydroxy[¹⁴C]cholesterol. 3 mg of microsomal protein is diluted to a volume of 5.3 ml with a potassium phosphate buffer (50 mM, pH 7.4) containing NaF (50 mM), cysteamine (31.4 mM), glucose-6-phosphate (12.7 mM) NADP (1.4 mM) and [¹⁴C]cholesterol (0.22 µCi, 55 Ci/mol). After a short pre-incubation (5 min at 37°C), the reaction is started by the addition of glucose-6-phosphate dehydrogenase suspension (7 µg). The reaction is stopped 1 h later by adding methanol (5 ml). The cholesterol and 7 α -hydroxycholesterol are extracted into a mixture of chloroform and methanol (2:1, v/v, 2 × 5 ml). The product and substrate are separated by thin-layer chromatography on silica gel eluting with toluene/ethyl acetate (3:7, v/v). The radioactive regions corresponding to cholesterol and 7 α -hydroxycholesterol are scraped off the plates and quantified by liquid scintillation counting. An alternative non-radioactive method to determine 7 α -hydroxylase activity is described in detail in Sect. M.2.0.8.

EVALUATION

Dose–response curves for standard and test drug are established using the serum LDL cholesterol value of the standard chow fed as the maximal effect and the cholesterol control data as zero.

CRITICAL ASSESSMENT OF THE METHOD

Potent drugs acting on cholesterol or bile acid absorption lower serum LDL cholesterol below the level measured in animals on standard chow. PPAR α agonists (fibrates) increase liver triglycerides in hamsters on a normal or high-fat diet (Planke et al. 1988). HMG-CoA reductase inhibitors (statins) are not well tolerated, the therapeutic range of statins which is effective against LDL cholesterol is very narrow, and the animals have to be treated for longer than 3 weeks to see the maximal effects of statins on serum LDL cholesterol.

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- polyoxy-ethylene phenol). Serum triglycerides increased 20-fold in a nearly linear fashion within 1 and 8 h and serum cholesterol levels increased sharply 2–3 times within 24 h (phase I). The hypercholesterolemia decreased nearly to control levels within the next 24 h (phase II). The test drugs employed or the vehicle for the controls were administered simultaneously with the Triton injection if only the acute effect of a drug was investigated or ad 1–3 days before. Serum triglyceride cholesterol analyses were carried out 0, 2, 4, 6, and 8 h after Triton injection in order to investigate drugs that interfere with triglyceride synthesis; and 0, 6, 24, and 48 h after Triton injection for investigation of drugs that are effective against cholesterol synthesis. In 1951 it was described by Kellner et al. that intravenous injection of nonionic detergents increased serum lipids up to 48 h. Later it was shown by Schotz et al. (1957) that the hyperlipidemia is induced by inhibition of triglyceride hydrolysis by lipoprotein lipase. Since then, lipolysis inhibition has been used to determine hepatic VLDL secretion rates. The VLDL secretion rate can be calculated from the increase in triglyceride and cholesterol over time after the Triton injection. Drugs interfering with hepatic triglyceride and cholesterol biosynthesis were shown to be active in phase I, while drugs interfering with cholesterol clearance, excretion and metabolism were active in phase II.

EVALUATION

Mean values \pm standard deviation are calculated for each group and time interval and compared statistically with the controls.

CRITICAL ASSESSMENT OF THE METHOD

The method employing Triton hypercholesterolemia is rather simple and rapid for detection of compounds interfering with the synthesis of triglycerides or synthesis and excretion of cholesterol. Since the test is rather artificial, the results have to be validated by other methods, e. g., *ex vivo* measurement of liver cholesterol synthesis.

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M.2.0.4

Triton-Induced Hyperlipidemia

PURPOSE AND RATIONALE

The systemic administration of the surfactant Triton to mice or rats results in a biphasic elevation of plasma cholesterol and triglycerides. Triton inhibits lipoprotein lipase (LPL) activity and so VLDL particle clearance (Frantz and Hinkelman 1955; Garattini et al. 1958, 1961; Holmes 1964; Tamasi et al. 1968; Millar et al. 2005).

PROCEDURE

Male Sprague Dawley or Wistar rats weighing 200–350 g were starved for 18 h and then injected intravenously with 200 mg/kg Triton WR 1339 (isooctyl-

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M.2.0.5

Fructose-Induced Hypertriglyceridemia in Rats

PURPOSE AND RATIONALE

Rats switched from a diet low in carbohydrates and high in protein to a high intake of fructose, develop an acute hypertriglyceridemia. Compounds are tested for inhibition of this phenomenon.

PROCEDURE

Male Sprague Dawley rats weighing 200–250 g were fed over a period of 1 week a diet enriched in protein with reduced carbohydrate content, e. g., Altromin C1080 or C1009. Groups of ten animals were treated for 3 days daily with the test compound or the standard (fenofibrate 30–100 mg/kg) or the vehicle (0–5% hydroxyl-ethyl-cellulose) by oral gavage. From the second to the third day water was withheld for a period of 24 h. Immediately afterwards, the animals were offered 20% fructose solution ad libitum for a period of 20 h. After this time, which was also 20 h after the last application of the test compound, the animals were anesthetized with isoflurane or CO₂/O₂ (70/30 v/v) and 1.2 ml blood was withdrawn by retro-orbital puncture. The blood was centrifuged at 6000 g for 5 min and the serum removed. The serum was analyzed for total cholesterol and triglycerides and glycerol using a colorimetric enzymatic assay for clinical diagnostics, as described in the previous chapter for rats (e. g. Roche Diagnostics).

EVALUATION

The average values of total glycerol for the treated groups are compared with those for the control group using ANOVA.

REFERENCES AND FURTHER READING

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M.2.0.6

Intravenous Lipid Tolerance Test in Rats

PURPOSE AND RATIONALE

Intravenous injection of a lipid emulsion results in an increase of triglycerides in serum. The lipolytic activity can be determined by measuring lipid elimination.

PROCEDURE

Male Wistar rats weighing 200–240 g are treated daily with various doses of the test compound or the vehicle over a period of 5 days. On the fifth day, two hours after the last administration of the test compound, the animals are anesthetized with isoflurane or 40–60 mg/kg sodium pentobarbital i.p. Then they are injected intravenously with 2 ml/kg of a 10% lipid emulsion (Intralipid Vitrum, Hausmann AG, St. Gallen, Switzerland). Prior to the injection and 10, 20, 30, and 40 min thereafter blood is withdrawn by retro-orbital puncture for determination of triglycerides.

EVALUATION

Peak levels as well as elimination constant and half life are determined.

REFERENCES AND FURTHER READING

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M.2.0.7**Influence on Lipoprotein-Lipase Activity****PURPOSE AND RATIONALE**

Post-heparin plasma lipolytic activity is measured by at least two lipase activities: hepatic lipase (HL) and lipoprotein lipase (LPL). Hypothyroid rats show a selective decline of post-heparin plasma hepatic lipase (Murase and Uchimura 1980).

For patients with familial lipoprotein lipase deficiency a rapid diagnostic test was developed (Gotoda et al. 1991). The method was used to study the influence of drugs on lipoprotein lipase activity (Tsumumi et al. 1993).

PROCEDURE

Male Wistar rats weighing 180–200 g receive either various single doses or one daily dose of test compounds over a period of several days. Blood samples are drawn from the tail vein into tubes containing 1 mg EDTA/ml. The animals are then injected with 100 U/kg heparin via the tail vein and blood samples are collected 5 min later. Plasma samples are used to determine lipoprotein lipase and hepatic lipase activity.

Lipoprotein lipase activity in postheparin plasma is measured using glycerol tri[1-¹⁴C]oleate as substrate and selective blocking of hepatic lipase activity with antiserum to rat hepatic lipase. Hepatic triglyceride lipase activity in postheparin plasma is obtained by subtracting lipoprotein lipase activity from total plasma lipase activity.

EVALUATION

The dose-related increase of plasma lipoprotein lipase activity and the time course after drug administration are evaluated.

REFERENCES AND FURTHER READING

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M.2.0.8**Influence on Several Steps of Cholesterol Absorption and Formation****PURPOSE AND RATIONALE**

Cholesterol levels in the body result from two sources: absorption from the gut and endogenous *de novo* synthesis. Certain natural products and the azetidinone drug ezetimibe inhibit cholesterol absorption and reduce plasma cholesterol levels in experimental animals and are therefore of potential pharmacologic interest in the treatment of hypercholesterolemia. In rodents cholesterol absorption can be decreased by inhibition of acyl coenzyme A:cholesterol acyltransferase (ACAT). In humans ACAT-inhibitors are not effective. Inhibition of cholesterol resorption results in a compensatory increase of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity, which can be decreased by (HMG-CoA) reductase inhibitors (statins). The hepatic cholesterol biosynthesis can be measured with several radioactive tracers as described later in Sect. M.3, by conversion of radioactive precursors into cholesterol. In addition to serum LDL cholesterol, hepatic cholesterol biosynthesis is a biomarker for the *in vivo* efficacy of cholesterol absorption inhibitors. Harwood et al. (1993) and van Hek and Davis (2002) studied the pharmacologic consequences of cholesterol absorption inhibition: alteration in cholesterol metabolism and reduction in plasma cholesterol concentration induced by a synthetic saponin β -tigogenin cellobioside or ezetimibe.

PROCEDURE

Male golden Syrian hamsters, weighing 100–120 g, are housed in a reversed light-dark cycle room (light between 3:00 P.M. and 3:00 A.M.) and receive a cholesterol-poor or cholesterol-enriched diet and water ad libitum 1 week prior to use. The animals are assigned to groups of 6 animals each and are given free access to water and chow that contains the compounds to be tested in appropriate concentrations for 4 days. At 9:00 A.M. of the fourth day of the study, the animals receive a 1.0-ml oral bolus of liquid formulation of cholesterol and radiolabeled cholesterol dissolved in Intralipid containing 15 mg of [³H]cholesterol (2.25 μ Ci) and 7.5 mg cholic acid for determining cholesterol absorption. At 9:00 A.M. of the fifth day (peak of the diurnal cycles of HMG-CoA reductase and cholesterol 7 α -hydroxylase activities) animals are anesthetized with pentobarbital and blood samples are obtained by cardiac puncture for deter-

mining plasma cholesterol and triglyceride levels, and cholesterol absorption.

Livers are removed, weighed, rinsed in 4°C saline, and apportioned for determining hepatic cholesterol levels, hepatic HMG-CoA reductase, cholesterol 7 α -hydroxylase activities, hepatic LDL receptor concentration, and cholesterol absorption.

For determination of total hepatic cholesterol, one aliquot of the liver tissue (0.5 g) is saponified in 5 ml ethanolic KOH, 3.3% (v/v) at 65°C for 1 h. After addition of 5 ml water and 2 mg 5-cholestene (internal standard), lipids are extracted with 15 ml petroleum ether. Samples are shaken several times to extract the sterols into the petroleum ether phase. Then 100 μ l of the petroleum ether phase (upper phase) is transferred into an HPLC autosampler vial and the solvent is removed by air-stream. For final HPLC analysis, the samples are mixed with an equal volume of HPLC eluent. The total cholesterol content is detected at 210 nm after isocratic HPLC separation on an RP C18 column (Merck Lichrospher 100, 250 \times 4 mm, 5 μ m) with 2-propanol-acetonitrile (55:45 v/v) as eluent solution (0.8 ml/min) using 5-cholestene as an internal standard.

Alternatively, hepatic cholesterol concentration can also be measured with a colorimetric enzymatic assay. Frozen samples of liver are thawed, homogenized and aliquots are extracted with chloroform/methanol and analyzed as described earlier in Sect. M.2.0.2 (Herling et al. 1999).

Hepatic microsomes for measurement of hepatic HMG-CoA reductase activity and cholesterol 7 α -hydroxylase activity are prepared according to Harwood et al. (1984) and Junker and Story (1985). For measurement of HMG-CoA reductase activity, 0.5 g liver are immediately homogenized at 4°C in 1 ml TEDK buffer (containing 50 mM Tris (pH 7.5), 1 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl) using 15 strikes in a Dounce homogenizer. For measurement of cholesterol 7 α -hydroxylase activity, 0.5 g liver pieces are immediately homogenized at 4°C in 1 ml PEDSKF buffer (containing 40 mM phosphate (pH 7.4), 5 mM EDTA, 5 mM DTT, 250 mM sucrose, 50 mM KCl, and 50 mM KF) using 20 strokes of a Dounce homogenizer. Homogenates are first centrifuged at 4°C for 20 min at 10,000 g and the resultant supernatant is then centrifuged at 4°C for 90 min at 178,000 g. The resulting microsomal pellets are resuspended in either 1.0 ml TEDK per g liver (HMG-CoA reductase determination) or 1 ml PEDSKF per g liver (cholesterol 7 α -hydroxylase determination) by

5 strokes of a Potter-Elvehjem pestle and are stored frozen in liquid nitrogen.

For measurement of hepatic HMG-CoA reductase activity, 50 μ g of microsomal protein is incubated for 30 min at 37°C in a final volume of 75 μ l of TEDK buffer containing 3.4 mM NADP⁺, 30 mM glucose-6-phosphate, 66.7 μ M [¹⁴C]HMG-CoA (10 cpm/pmol), 15000–20,000 cpm [³H]mevalonate (0.6–1.2 Ci/mmol) as an internal standard, and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. After incubation, 10 μ l of 6 M HCl are added to terminate the enzyme reaction and to convert the newly formed mevalonate to mevalonolactone. The mevalonolactone is then separated from unreacted substrate by silica gel thin-layer chromatography. After development in toluene-acetone 1:1, the region of the chromatogram corresponding to R_f = 0.4–1.0 is removed, immersed in liquid scintillation fluid, and counted using a dual channel ³H/¹⁴C program. HMG-CoA reductase activity is expressed as pmoles of mevalonate formed from HMG-CoA per min of incubation at 37°C per mg of microsomal protein.

Cholesterol 7 α -hydroxylase activity was determined with a modified method according to the procedure of Hylemon (1989) with the following modifications. To measure 7 α -hydroxylase activity, 2 mg microsomal protein (1 ml in assay buffer) is pre-incubated for 10 min at 37°C with 20 μ l cholesterol solution (1 mg/ml) in 45% hydroxypropyl- β -cyclodextrin (ICN) in order to saturate the enzyme with cholesterol substrate. 7 α -Hydroxycholesterol is measured with isocratic HPLC analysis on a C-18 reversed-phase column (Merck 250 \times 4 mm, 5 μ m), at 240 nm with acetonitrile:methanol (70:30 v/v) as eluent (0.7 ml/min) using 7 β -hydroxycholesterol as internal standard. The protein content of the microsomes is measured by the BCA method (Pierce).

Cholesterol 7 α -hydroxylase activity is expressed as pmoles of 7 α -hydroxycholesterol formed from cholesterol per minute of incubation per milligram of microsomal protein.

Hepatic *in vivo* cholesterol biosynthesis is determined with a method according to Dietschy and Spady (1984). [¹⁴C]Octanoic acid sodium salt (NEC 092H, concentration 0.1 mCi/ml in ethanol, NEN, Cologne, Germany) is used as tracer. The metabolism of the tracer into liver cholesterol is investigated using the following procedure. The solution is concentrated about 20-fold under a nitrogen stream and dissolved in 0.9% NaCl to a final concentration of 50 μ Ci/ml. This

solution is injected into the femoral vein at a dose of 10 $\mu\text{Ci}/100\text{ g}$ body weight under ether anesthesia.

The animals are killed 1 h after administration and the liver removed and washed in isotonic NaCl solution. Three aliquots (0.5 g) are extracted in 30 ml of 6.6% ethanolic KOH at 65°C for 2 h. After cooling down to room temperature, 5 ml aqua is added and the cholesterol is extracted twice with 15 ml petroleum ether into a volumetric flask. The petroleum ether is removed under an air stream. The lipid phase is resolved and transferred three times with 2 ml ethanol acetone 1:1 (v/v) into a 15-ml glass vial after heating the glass container used. The cholesterol is precipitated with digitonin after adding one drop of 1M HCl, 2 ml 10% aqueous aluminum chloride, 3 ml 0.5% digitonin in ethanol water 1:1 (v/v), and incubated for 1 h at 45°C in a water bath. The liquid phase is discarded after centrifugation. The pellet is washed with 5 ml acetone three times and once with ether. Each pellet is rolled out by hand on a table until completely dry.

To cleave the digitonin–cholesterol complex, the vials are heated to 80°C and the cholesterol resolved with 0.6 ml pyridine. When the samples are cooled to room temperature, 5 ml ether is added twice, the samples are centrifuged, and the liquid phase is transferred into scintillation vials. The ether is removed under air stream, and the residual cholesterol resolved with 1 ml methanol and 10 ml scintillation cocktail (Ecolite ICN, Eschwege, Germany).

The radioactivity is counted in a β -scintillation counter. The disintegrations per minute (DPM) are calculated per gram of liver tissue after background correction and correlated with the liver cholesterol concentration in a graph plotting liver cholesterol on the *x*-axis and radioactivity (dpm/g liver tissue) on the *y*-axis. Orientation of treated groups relative to cholesterol-fed and non-cholesterol-fed control animals gives information about sterol turnover.

Hepatic LDL receptor levels are measured by enzyme immune blotting (Cosgrove et al. 1992). One hundred–150 μl of the soluble extracts containing 100–600 μg protein are adjusted to 2% SDS and 0.2 M sucrose by addition of 0.33 volumes of electrophoresis sample buffer (containing 320 mM Tris (pH 6.8), 8% SDS, and 0.8 M sucrose). Mixtures are applied without heating and without addition of β -mercaptoethanol to 6-mm wells of a 7.5% (w/w) 0.1% SDS-containing polyacrylamide slab gel of 1.5 mm thickness. Electrophoresis is conducted at room temperature with a constant current of 15 mA/gel. Prestained molecular weight markers are included in a separate lane to monitor separation. After electrophoresis, proteins

migrating into the gel are electrophoretically transferred to S&S BA85 nitrocellulose membranes at 18°C with a constant voltage of 120 V for 6–8 h in 25 mM Tris, 192 mM glycine buffer (pH 8.3), containing 20% methanol.

After transfer, the nitrocellulose paper is incubated with 100 ml of Tris buffered saline containing 3% gelatin for 30 min at room temperature with gentle shaking. After incubation, the nitrocellulose sheet is removed from the blocking solutions, immersed without rinsing into 50 ml Tris buffer solution (TBS) containing 1% gelatin and 250 μl of anti-LDL receptor peptide antiserum (final dilution 1:200) and incubated for 2 h at room temperature with gentle shaking. The nitrocellulose sheet is washed twice for 10 min each with TBS containing 0.05% Tween-20 and once for 10 min with TBS. The washed nitrocellulose sheet is incubated with 50 ml TBS containing 1% gelatin and 100 μl of goat anti-rabbit IgG-horseradish conjugate (final dilution 1:500) at room temperature for 1 h with gentle shaking. After incubation, the nitrocellulose is washed again as described above. During this final wash, 40 mg of 4-chloro-1-naphthol is dissolved in 10 ml methanol, and 50 μl cold 30% hydrogen peroxide is added to 50 ml TBS containing 1% gelatin. After draining the final TBS wash from the nitrocellulose, the two solutions are mixed and immediately added to the nitrocellulose. The mixture is incubated at room temperature with gentle shaking until the desired color development is observed. The nitrocellulose sheet is washed with running tap water for 15 min. The nitrocellulose is dried between pieces of filter paper and the intensity of color formation is quantitated by reflectance densitometry using a suitable instrument (e. g., Hoefer Scientific Instruments GS300 Transmittance/Reflectance Scanning Densitometer). Color intensity, which is a linear function of the number of LDL receptors in the analyzed fraction, is expressed in terms of mm peak height (arbitrary reflectance units).

Intestinal cholesterol absorption is estimated with the dual isotope feces ratio method using [^3H]sitostanol and [^{14}C]cholesterol. Each hamster received an intragastric dose of a mixture of [^3H]sitostanol and [^{14}C]cholesterol dissolved in Intralipid or plant oil, or in detergent mix (Tricaprin/Tricapril). The feces is collected for 48 h after dosing and homogenized with water; aliquots are oxidized in a sample oxidizer, which traps CO_2 and H_2O in separate vials mixed with liquid scintillation fluid, and counted using a background correction program. The absorption ratio can be calculated by changes of the $^{14}\text{C}/^3\text{H}$ ratio in application solution and feces with

the following formula ($^{14}\text{C}/^3\text{H}$ application solution – $^{14}\text{C}/^3\text{H}$ feces) / $^{14}\text{C}/^3\text{H}$ application solution.

If a sample oxidizer is not available an alternative method is the recovery of radioactivity present in the liver and plasma 24 h after an oral bolus of [^3H]cholesterol. For assessment of hepatic radioactivity, 1.0-g liver pieces are placed in 50-ml polypropylene tubes and incubated with 2.5 ml of 2.5 M KOH for 2 h at 75°C. After allowing the saponification mixture to cool to room temperature, 5.0 ml of 80% ethanol, 0.1 ml [^{14}C]cholesterol (40,000 dpm, 15 dpm/nmol) as carrier and extraction standard and 10 ml of hexane are added. Tubes are capped and shaken vigorously for 1 min. Mixtures are permitted to stand to allow phase separation, and are shaken vigorously for an additional minute. Mixtures are centrifuged at 1000 g for 5 min. Duplicate 3.0-ml aliquots of each hexane layer are removed, transferred to 20-ml liquid scintillation vials, mixed with 10 ml liquid scintillation fluid, and counted using a dual channel $^3\text{H}/^{14}\text{C}$ program.

After correction for recovery losses, hepatic radioactivity is calculated based on total liver weights. For assessment of total plasma radioactivity, 200- μl aliquots of plasma are added to 20-ml liquid scintillation vials and decolorized by addition of 25 μl of hydrogen peroxide. Then 10 ml aqueous scintillation fluid is added and the mixture assessed for radioactivity. Total radioactivity in the plasma is calculated based on the assumption that hamsters possess approximately 4.0 ml plasma per 100 g body weight. The degree of cholesterol absorption is expressed as a percentage of the total radioactivity administered that is present in the liver plus plasma 24 h after bolus administration.

For **determination of bile acids in feces**, pooled feces from each group of animals is collected over a period of 48 h (about 20 g). After addition of 9 ml ethanol, 1 ml water and hyodeoxycholate (internal standard) the mixture is continuously shaken at 90°C overnight. Samples are centrifuged and an aliquot of the liquid phase is diluted 1:5 with methanol for quantification of bile acids. Individual bile acids (deoxycholate; lithocholate; 12-oxo lithocholate) are separated and quantified by HPLC-MS (HPLC: RP C18 column Merck PurospherTM, end-capped, 5 μm , 125 \times 3 mm; gradient elution (20%–60% eluent B, 15 min, 0.6 ml/min; eluent A: 90% H₂O, 10% acetonitrile; eluent B: 10% H₂O, 90% acetonitrile, buffered at pH 8 with 20 mM ammonium acetate; MS detection: electrospray negative mode Hewlett Packard, 1100 MSD).

For **lipid and lipoprotein determinations**, serum or plasma can be used. Blood samples are treated either with EDTA or heparin to prevent clotting or without an anticoagulant and are then centrifuged, as described earlier in Sects. M.2.0.2 and M.2.0.3.

EVALUATION

Inhibition of cholesterol resorption results in a decrease of hepatic cholesterol and a compensatory increase of hepatic HMG-CoA reductase activity resulting from de novo cholesterol synthesis and an increase of hepatic LDL receptor levels.

MODIFICATIONS OF THE METHOD

Ogishima and Okuda (1986) described an improved method for assay of cholesterol 7α -hydroxylase activity. Cholesterol 7α -hydroxylation is performed in liver microsomes utilizing cholesterol as substrate. 7α -Hydroxycholesterol is converted by the action of cholesterol oxidase into 7α -hydro-4-cholesten-3-one having an intense absorption at 240 nm.

Hylemon et al. (1989) reported a modified HPLC-spectrophotometric method for measuring cholesterol 7α -hydroxylase activity by using a C-18 reverse-phase column to separate 7α -hydroxy-4-cholesten-3-one and 4-cholestene-3-one and by adding 7β -hydroxycholesterol to each reaction mixture as an internal recovery standard. This method measures simultaneously cholesterol 7α -hydroxylase activity using endogenous cholesterol and exogenous [$4\text{-}^{14}\text{C}$]cholesterol as substrate.

Princen and Meijer (1990) measured cholesterol 7α -hydroxylase activity in cultured rat hepatocytes.

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M.3

Inhibition of Cholesterol Biosynthesis

M.3.1

General Considerations on Cholesterol Biosynthesis

The following steps are involved in cholesterol biosynthesis:

- HMG-CoA synthase (hydroxymethylglutaryl-coenzyme A synthase)
- forming **hydroxymethylglutaryl-CoA** from acetyl-CoA and acetoacetyl-CoA
- HMG-CoA reductase (hydroxymethylglutaryl-coenzyme A reductase)
- forming **mevalonic acid** from hydroxymethylglutaryl-CoA.

Inhibition of cholesterol synthesis on this step is used at present successfully for therapy (see below)

- Mevalonate kinase forming **5-phospho-mevalonic acid** from mevalonic acid,
- Phospho-mevalonate kinase forming **5-pyrophospho-mevalonic acid** from 5-phospho-mevalonic acid,
- Pyrophospho-mevalonate decarboxylase forming **3-isopentyl-pyrophosphate** from 5-pyrophospho-mevalonic acid,
- Isopentyl-pyrophosphate isomerase forming **3,3-dimethyl-pyrophosphate** from 3-isopentyl-pyrophosphate,

- Dimethylallyl-transferase forming in a two step process **geranyl pyrophosphate** from 3,3-dimethyl-pyrophosphate, and
- **Farnesyl pyrophosphate** from geranyl pyrophosphate,
- Squalene synthetase forming **squalene** from farnesyl pyrophosphate,
- Squalene epoxidase forming **2,3-oxidosqualene** from squalene,
- 2,3-Oxidosqualene cyclase forming **lanosterol** from 2,3-oxidosqualene.

Following the formation of lanosterol (4,4,14 α -trimethylcholesta-8(9),24-dien-3 β -ol), a series of enzyme reactions is required to produce cholesterol (Bae et al. 1999):

- to 4,4,14 α -trimethylcholesta-8(9)-en-3 β -ol,
- to 4,4-dimethylcholesta-8(9),14-dien-3 β -ol,
- to 4,4-dimethylcholesta-8(9)-en-3 β -ol,
- to cholesta-8(9)-en-3 β -ol,
- to cholesta-7-en-3 β -ol,
- to cholesta-5,7-dien-3 β -ol (7-dehydrocholesterol), and finally
- to cholesta-5-en-3 β -ol (cholesterol) by 7-dehydrocholesterol reductase.

Besides **inhibition of HMG-CoA reductase** (detailed description below) other approaches to inhibit biosynthesis of cholesterol are reported:

- **inhibition of HMG-CoA synthase** (Goldstein and Brown 1990; Miller et al. 1980; Greenspan et al. 1987; Grayson and Westkaemper 1988);
- **inhibition of squalene synthase** (Ness et al. 1994; Sliskovic and Picard 1997; Rosenberg 1998; Hiyoshi et al. 2000; Squalene synthase plays an important role in sterol biosynthesis by catalyzing the head-to-head condensation of two molecules of farnesylpyrophosphate. The enzyme is a single 47,000 Da polypeptide that is bound to the subcellular membranes of the endoplasmic reticulum in yeast and mammalian liver (see below);
- **inhibition of squalene epoxidase** (Ryder 1992); Squalene epoxidase catalyzes the conversion of squalene to (3S)-2,3-oxidosqualene, an essential step in the biosynthesis of sterols in mammals, plants, and microorganisms (see below)
- **inhibition of 2,3-oxidosqualene cyclase** (Gerst et al. 1986; Cattel et al. 1989; Sen and Prestwich 1989; Dollis and Schuber 1994; Mark et al. 1996; Eisele et al. 1997; Morand et al. 1997; Abe et al.

1998a); The enzyme 2,3-oxidosqualene cyclase is of special interest due to its dual function: cyclization of 2,3-monoepoxysqualene to lanosterol and 2,3:22,23-diepoxy-squalene to oxysterol. An orally active oxidosqualene:lanosterol cyclase inhibitor (Ro48–8071) showed potent noncompetitive inhibition of bacterial squalene:hopene cyclase from *Alicyclobacillus acidocaldarius*. A tritium-labeled isotopomer of this nonterpenoid inhibitor, which possesses a benzophenone photophore, was chemically synthesized as a photoaffinity label (Abe et al. 1998b);

- **inhibition of 7-dehydrocholesterol reductase** (Amin et al. 1996); Deficiency of this enzyme causes a severe developmental disorder with multiple congenital and morphogenic abnormalities, the Smith-Lemli-Opitz syndrome (Waterham and Wanders 2000).

Search and development of new cholesterol-lowering drugs is continued (Abe and Prestwich 1998). Whether the efficacy and safety of these agents in man will be on the long run superior to currently available therapies remains to be determined.

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M.3.2

Determination of HMG-CoA-Reductase Inhibitory Activity

M.3.2.1

General Considerations on HMG-CoA-Reductase

More than 70% of the total production of body cholesterol in humans is derived from de novo synthesis. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate limiting enzyme governing cholesterol biosynthesis and the synthesis of other isoprenoids in mammalian cells (Rodwell et al. 1976).

The development of HMG-CoA reductase inhibitors (called statins) offers an advance in the treatment of hypercholesterolemia by interfering with the crucial step of cholesterol biosynthesis. When inhibiting hepatic HMG-CoA reductase, the inhibitors trigger an increased production of LDL receptors in the liver. As LDL receptor activity increases, more LDL is extracted from the blood and thus the level of circulating LDL-cholesterol is reduced. Pharmacological evaluation of HMG-CoA reductase inhibitors is based on studies on the inhibition of the isolated enzyme HMG-CoA reductase *in vitro*, on the inhibition of the incorporation of ¹⁴C sodium acetate into cholesterol in isolated liver cells, and on the effect of HMG-CoA reductase inhibitors *in vivo*. HMG-CoA reductase activity has a diurnal rhythm in liver and intestine which has to be considered for *in vivo* studies (Shapiro and Rodwell 1969; Shefer et al. 1972).

HMG-CoA reductase inhibitors have been associated with skeletal myopathy (rhabdomyolysis) in humans and experimental animals (Flint et al. 1997; Klotz 2003). The rhabdomyolysis is probably mechanism based, from the cerivastatin cases it could be concluded that the risk of rhabdomyolysis is related to active drug levels in the systemic circulation and peripheral tissues (Graham et al. 2004).

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M.3.2.2

Inhibition of the Isolated Enzyme HMG-CoA-Reductase in Vitro

PURPOSE AND RATIONALE

For screening purposes, studies on the inhibition of HMG-CoA reductase obtained from rat liver microsomal fraction can be used (Avigan et al. 1975; Philipp and Shapiro 1979).

PROCEDURE

The inhibitory activity of the test compound on HMG-CoA reductase is estimated with soluble enzyme preparations obtained from the microsomal fraction of rat liver (Philipp and Shapiro 1979). The enzyme reaction is carried out with 50 μ l partially

purified HMG-CoA reductase in buffer containing 25 mM Tris, 10 mM EDTA, and 10 mM dithiothreitol at pH 7.5, 20 μ l of 910 μ M HMG-CoA solution containing 100 nCi (3.7 KBq) of 14 C-HMG-CoA and 20 μ l of NADPH regenerating system (5.2×10^{-2} M glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 5.3×10^{-3} M NADP), with the actual concentration of 50 mM NADPH. The final incubation volume is 200 μ l. The main reaction is preceded by 20 min preincubation with the NADPH regenerating system at 37°C, followed by 20 min incubation at 37°C of the completed samples with the test compound or the standard and stopped by addition of 75 μ l 2 N HClO₄. After 60 min at room temperature, the samples are cooled in an ice-bath and neutralized by addition of 75 μ l 3 N potassium acetate. Supplementing the volume with water to 500 μ l, the precipitate is centrifuged and 250 μ l of the clear supernatant are applied to a column (0.6 \times 8.0 cm) of BIORAD AG 1-X8 (100–200 mesh). Mevalonolactone is eluted with water discarding the first 750 μ l and collecting the next 3500 μ l. Five hundred μ l of the eluate are used for measurement in duplicate, mixed in vials with 10 ml Quicksint (Zinsser) and measured in a liquid scintillation counter (Beckman). The assay is generally performed in triplicate. Lovastatin sodium is used as standard.

EVALUATION

The mean values with and without inhibitors are compared for the calculation of inhibition. IC_{50} values are calculated.

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M.3.2.3

Inhibition of the Incorporation of 14 C-Sodium Acetate into Cholesterol in Isolated Liver Cells

PURPOSE AND RATIONALE

De novo synthesis of cholesterol from labelled acetate can be measured in isolated liver cells. This synthesis can be inhibited by incubation with HMG-CoA reductase inhibitors.

PROCEDURE

Monolayers of primary cell cultures from hepatocytes of female rats or of cultures of HEP-G2 cells (human hepatoma cells) are incubated in a lipoprotein deficient medium for 1 h with various concentrations of the potential HMG-CoA reductase inhibitor or the standard (Lovastatin). Thereafter, the labelled precursor 14 C sodium acetate is added to the medium and the incubation continued for further 3 or 24 h. To one part of the cells an internal standard of 3 H-cholesterol is added and the cells are saponified by alkaline. The lipids of these saponified cells are extracted with chloroform/methanol. The lipid mixture is separated by preparative thin layer chromatography. The localization of cholesterol is identified by exposure to iodine vapor and the amount of newly synthesized 14 C-cholesterol is determined in a scintillation counter.

Cell protein is determined in another part of the cells. A third part of the cell culture is used to control the integrity of the cells by light microscopy and tested biologically by determination of the release of lactate dehydrogenase into the incubation medium.

EVALUATION

The amount of newly synthesized cholesterol per mg cell protein is calculated. The inhibitory capacity of the test drug is estimated against a solvent control. IC_{50} values are calculated from the results of various concentrations.

MODIFICATIONS OF THE METHOD

Chen and Kandutsch (1976) studied the effects of cholesterol derivatives on sterol biosynthesis in cultures of various cell types.

Liver specificity of inhibition of sterol synthesis is studied by *in vitro* uptake into hepatocytes and human

skin fibroblasts (Scott 1990) or in slices of various tissues after oral treatment with HMG-CoA reductase inhibitors (Tsujiita 1990).

Shaw et al. (1990), however, caution against the use of HEP-G2 cells which may not be the cell system of choice to demonstrate liver selectivity of inhibitors of HMG-CoA reductase.

Cultures of HEP-G2 cells have also be used to test inhibition of cytoplasmic acetoacetyl-CoA thiolase (Greenspan et al. 1989) or inhibition of squalene epoxidase (Hidaka et al. 1991).

Pearce et al. (1992) used HEP G2 cells to study the hypocholesterolemic activity of synthetic and natural tocotrienols *in vitro*.

Raiteri et al. (1997) investigated drugs acting at different steps of the mevalonate pathway on arterial smooth muscle cell proliferation. Competitive HMG-CoA reductase inhibitors dose-dependently decreased smooth muscle cell proliferation.

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M.3.2.4

Ex Vivo Inhibition of Cholesterol Biosynthesis in Isolated Rat Liver Slices

PURPOSE AND RATIONALE

Inhibition of cholesterol biosynthesis in rat livers can be measured in an *ex vivo* assay after oral treatment with HMG-CoA reductase inhibitors by cholesterol synthesis from labelled sodium octanoate.

PROCEDURE

Male Sprague Dawley rats weighing 110–130 g are kept on a reverse light cycle (lights 3:00 P.M. to 3:00 A.M.) for 14 days prior to use. Throughout the period of adaptation, the rats have free access to a low cholesterol diet and tap water. On the day of the experiment, the test compounds are given orally between 9:00 and 11:00 A.M. as suspensions in 0.5% methylcellulose. After one hour, the rats are sacrificed, the livers removed and transferred to chilled oxygenated Krebs-Ringer-bicarbonate buffer (pH 7.4). The livers are then chopped into 0.8-mm² pieces using a McIlwain tissue chopper (e. g., Brinkmann Instr., Westbury, USA) and are suspended in the same buffer. Aliquots of the suspension are pipetted, in triplicate, into culture tubes which contain [¹⁴C]sodium octanoate (300 μM/l, 6.67 Ci/M). The assay volume is 1 ml. The tubes are gassed with 95% O₂/5% CO₂ for 10 s, stoppered with a serum cap, and incubated at 37°C in a metabolic shaker at 150 oscillations/min for 90 min.

The reaction is stopped by addition of 1 ml 15% KOH in ethanol. An aliquot of the mixture is assayed for protein concentration. An internal standard [³H]cholesterol (30,000 dpm) is added to determine recovery, which ranges from 70–80%. The tubes are saponified at 75°C for 2 h and then extracted with 10 ml of petroleum ether for 30 min. The lower aqueous phase is frozen in a dry ice/alcohol mixture, and the ether phase is removed, washed with 2 ml glass-distilled water and then evaporated to dryness. The [¹⁴C]cholesterol synthesized is separated by thin layer chromatography on plastic silica gel plates using chloroform as eluent. After visualization with iodine, the

cholesterol spots are cut out, and radioactivity quantitated by liquid scintillation counting.

EVALUATION

Results are expressed as percentage inhibition compared to vehicle-treated control values. Using various doses, ED_{50} values of inhibition can be calculated from dose-response curves.

MODIFICATIONS OF THE METHOD

Koga et al. (1990) tested the tissue-selective inhibition of cholesterol synthesis *in vivo* by pravastatin sodium in mice. Drugs were orally administered to mice 2 h prior to an intraperitoneal injection of [14 C]acetate. The animals were sacrificed 1 h afterwards and the cholesterol synthesis determined in various organs.

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M.3.2.5

Effect of HMG-CoA-Reductase Inhibitors *in Vivo*

PURPOSE AND RATIONALE

A strain of rabbits with heritable hyperlipidemia, the WHHL strain, has been described by Watanabe et al. (1980, 1985, 1988). These animals develop digital xanthoma and aortic and coronary atherosclerosis already at an early age. This animal is considered to be a suitable model for the evaluation of preventive or even regressive effects of drugs on hyperlipidemia and atherosclerosis.

PROCEDURE

Male heterozygous WHHL rabbits weighing 1.8 to 2.5 kg at an age between 8 and 20 weeks are used. The animals are housed individually under standard conditions (standard rabbit diet and water ad libitum) and are allowed to accommodate 2 weeks prior to treatment. The test compounds are suspended in 0.5% methylcellulose and are administered each day orally by gavage in the afternoon to insure an increased plasma level at night, since in man HMG-CoA reductase activity has been found to be higher at night than during daytime (Shapiro and Rodwell 1969; Sheffer et al. 1972) similar to the enzyme in rodents. The treatment is continued for 14 days.

Blood samples are taken in the morning without previous feeding. Two ml of blood are drawn from the outer ear vein 5 days prior to the beginning of treatment, on days 3 and 8 of treatment and 30 days after the end of treatment for the determination of biochemical parameters. In addition, 6 ml blood are drawn at the first and the last day of treatment and 10 days after the end of treatment for determination of biochemical parameters and lipoprotein profile. In order to obtain serum, blood is allowed to clot at room temperature and then centrifuged twice at 10,000 rpm.

The following biochemical parameters are determined in non-frozen samples (kept at 4°C): total cholesterol, HDL-cholesterol, triacylglycerol, as well as creatinine, total bilirubin, alkaline phosphatase, alanine amino transferase (ALAT), aspartate amino transferase (ASAT), and γ -glutamyl transferase (γ -GT) using commercially available kits.

The separation of serum lipoproteins by gel permeation chromatography is performed according to Ha and Barter (1985). This method is particularly well suited for the metabolic studies of lipoproteins, because the elution profile can be obtained from the same sample under more gentle conditions than by sequential ultracentrifugation. According to their descending particle size, the elution profile of lipoproteins in the same fraction of density (<1.21 g/ml) shows, as the elution progresses, three major peaks which correspond to very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), respectively.

Since the amount of serum collected from one rabbit is too low to determine the lipoprotein profile from each sample, the total lipoproteins are isolated by ultracentrifugation at a density <1.21 g/ml and the lipoproteins thus obtained in each group are pooled two by two prior to injection onto the cross-linked agarose HR 50 column (Superose 6B). As a result,

each lipoprotein profile represents the lipoprotein size distribution of an equal volume of lipoproteins obtained from two rabbits. Cholesterol and triacylglycerol concentrations are determined in each 1 ml elution fraction.

EVALUATION

The data at 5 days before beginning of treatment and of day 0 of each animal are pooled and the mean is taken as reference value. Student's paired *t*-test is used to calculate for each group the significance of difference between mean values.

MODIFICATIONS OF THE METHOD

Extension of the treatment period up to 24 weeks allows to evaluate the meanwhile apparent atherosclerotic lesions in the aorta and the coronaries by gross observation and light microscopy (Tsujita 1990).

Kasim et al. (1993) studied the effect of lovastatin on the secretion of very low density lipoprotein lipids and apolipoprotein B in the Zucker obese rat which is basically a model for genetic hypertriglyceridemia.

Soma et al. (1993) studied the effects of HMG-CoA reductase inhibitors on carotid intimal thickening induced by placing a nonocclusive, biologically inert, soft, hollow Silastic collar around both carotid arteries in normocholesterolemic rabbits (Booth et al. 1989).

Bocan et al. (1994) assessed atherosclerotic lesion development in the thoracic artery and chronically denuded iliac-femoral artery of hypercholesterolemic New Zealand White rabbits using inhibitors of HMG-CoA reductase.

Krause and Newton (1995) tested the lipid-lowering activity of atorvastatin and lovastatin in several rodent species (rat models, guinea pigs, rabbits). They concluded that normal rats can be used as a preclinical tool to assess the efficacy of HMG-CoA reductase inhibitors since triglyceride lowering correlates with cholesterol lowering in LDL animal models.

Johnston et al. (2001) induced atheroma formation in the aortas of C57BL/6 mice by long-term administration of Poloxamer-407, a nonionic surfactant, and tested the potency of selected statin drugs in this model of hyperlipidemia and atherosclerosis.

Delsing et al. (2003) studied differential effects of a calcium antagonist and a statin and their combination on atherosclerosis in ApoE*3-Leiden transgenic mice.

Aoki et al. (2002) induced postprandial lipemia in rats by administration of a lipid emulsion and measured the triglyceride-lowering effect of a statin.

Ugawa et al. (2002) established an experimental model of the escape phenomenon in hamsters in which plasma cholesterol, initially reduced by statins, increases again on long-term administration, and tested the effectiveness of a squalene synthase inhibitor in this model.

Yokota et al. (2003) tested the protective effect of a HMG-CoA reductase inhibitor on experimental renal ischemia-reperfusion injury in mice.

Abletshauer et al. (2002) reported a new method for biosensing of arteriosclerotic nanoplaque formation and interaction with an HMG-CoA reductase inhibitor and recommended this method for experimental and clinical studies.

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M.3.2.6

Influence of Statins on Endothelial Nitric Oxide Synthase

PURPOSE AND RATIONALE

Several studies indicate that the beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) on endothelial function and cardiovascular ischemic events may be attributed not only to their lipid-lowering effect but also to direct effects on the atherosclerotic vessel wall. One such effect is the upregulation of nitric oxide synthase, which generally leads to vasorelaxation (Kano et al. 1999; Amin-Hanjani et al. 2001; Rikitake et al. 2001; Sata et al. 2001; Sumi et al. 2001; Jones et al. 2002; Laufs et al. 2002; Mitani et al. 2003a, 2003b; Parker et al. 2003; Wassmann and Nickenig 2003; Yamamoto et al. 2003; Kumai et al. 2004).

Parker et al. (2003) compared different statins for e-NOS subcellular localization, formation of pro-oxidants, and endothelial-dependent vascular function.

PROCEDURE

Endothelium-Dependent Vascular Relaxation Ex Vivo in Statin-Treated Rats

Sprague Dawley rats (~160 g) on standard chow are treated with HMG-CoA reductase inhibitors for 8 days at doses that do not result in plasma cholesterol reductions as determined in previous experiments. To as-

say vascular relaxation, aortas are dissected out and perivascular tissue removed. Aortic rings (~5 mm) are incubated at 37°C in pH 7.4 Krebs-bicarbonate buffer with 5 mM glucose and 95% O₂/5% CO₂, and suspended on a strain gauge transducer to measure isometric circumferential tension. After adjustment basal tension to 2.0 g over 90 min, aortic rings are contracted submaximally with a thromboxane receptor agonist (U-46619) and exposed to increasing concentrations of acetylcholine (or nitroprusside as control), which is known to induce endothelium-dependent relaxation by activating e-NOS.

Data are expressed as mean (±SEM) percentage decrease in tension from the level of induced tone. Significance is determined with Student's *t*-test/two-way ANOVA.

Vascular Endothelial Cell Culture

Human arterial endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) are used at passages 3–8. Culture and statin incubations are conducted in Clonetics complete EGM medium (M-188-derived) with 2% serum. For experiments using redox probes, cells are grown in 24-well plates or collagen-coated glass chamber-slides to near confluency. For cell-based assays using oxyhemoglobin and cytochrome c, endothelial cell cultures are pretreated with statins in flasks, then harvested by brief trypsinization, centrifuged and suspended in KREBS-Ringer buffer with 5.5 mM glucose and 95% O₂/5% CO₂ for assay in cuvettes.

Fluorescence Assays for Intracellular Reactive Nitrogen and Oxygen Species

As peroxynitrite indicator DHR-123 is used, which is non-fluorescent and enters cells readily where it is oxidized (with limited selectivity by [•]ONOO and [•]OH) to the cationic, fluorescent rhodamine-123 (RH-123), which is entrapped in mitochondria (Kelm et al. 1997). Statins are added to endothelial cell cultures for 4–16 h followed by addition of DHR-123 (5 μM), bradykinin (1 μM) and A23187 (1 μM) to activate e-NOS. At the indicated times, cells are washed with PBS, and intracellular fluorescence is assayed using a fluorescent plate reader (Cytofluor-4000, λ_{ex} = 485 ± 20, λ_{em} = 530 ± 25 nm).

DHR oxidation is also assayed *ex vivo* in aortic specimens isolated from statin-treated rats. Aortic rings are incubated in 300 μl of EGM medium containing DHR-123 (5 μM), bradykinin (1 μM) and A23187 (1 μM) at 37°C for 90 min. Samples are washed twice in PBS, the homogenized in PBS + 0.1% Tween-20 +

0.1% SDS, and duplicate aliquots are taken for fluorescence readings. Protein is determined by the Bradford method. A fluorescence standard curve for DHR-123 oxidation product (HR-123) is generated and results are calculated as pmol product per mg protein.

Spectrophotometric Assay for Superoxide Anion

The assay of superoxide anion in the presence of NO in intact cells is accomplished by an adaptation of the spectrophotometric method described by Kelm et al. (1977). Endothelial cells are treated with statins in monolayer culture, then detached and resuspended in Krebs-Ringer buffer. Cells are added to cuvettes (7.5×10^5 to 1.25×10^6 cells/ml) with oxy-hemoglobin ($3 \mu\text{M}$), ferricytochrome c ($10 \mu\text{M}$) and CaCl_2 (0.1 mM) and assayed at 37°C with continuous stirring and 95% $\text{O}_2/5\%$ CO_2 flow. Agonists are added at 5-min intervals. Absorbance spectra from 400 to 600 nm are collected at 1-min intervals using a Beckman DU-7500 diode array spectrophotometer with data capture software. The superoxide anion-specific reduction of cytochrome c is measured as the change over time in absorbance difference between 465 nm and the isosbestic wavelength 525 nm.

Fluorescence Microscopy with DHR-123 and e-NOS Immunofluorescence

Human arterial endothelial cells are grown on glass chamber slides and incubated for several hours with the statins and $10 \mu\text{M}$ DHR-123. Localization of the DHR-123 oxidized product is analyzed by epifluorescence using Nikon Microphot-FXA with Chroma High-Q resorufin filter and interference contrast optics. Images are captured using a Photometric Image Point CCD video camera. For e-NOS localization, human arterial endothelial cells are fixed and processed for immunofluorescence using rabbit polyclonal anti-eNOS antibody at $2 \mu\text{g/ml}$ and LRSC-conjugated donkey anti-rabbit IgG second antibody (Jackson ImmunoResearch). Images comparing control and treated cells are obtained using the same CCD exposure times and video amplifier gain.

Subcellular Fractionation and Immunoblotting of Endothelial Cells

Human umbilical vein endothelial cells are incubated with statins followed by harvesting by scraping into 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, pH 7.4, with protease inhibitors (Sigma, P-8340), and lysed by brief sonification in a glass homogenizer. Cell lysates are cleared at 500 g and fractionated by centrifugation at 16,000 g. The 16,000 g pellet is resuspended

and ultracentrifuged in a discontinuous sucrose gradient (20 mM Tris-HCl, 1 mM EDTA, pH 7.4, with steps of 0.50 and 1.12 M sucrose) to isolate plasma membranes and Golgi/mitochondria-rich intracellular membranes. Microsomes and cytosol are isolated from the 16,000 g supernatant. Samples are assayed for e-NOS protein level by SDS-PAGE (4%–20% gradient gels) and western transfer to PVDF membranes (Novex LC-2002), and probed using rabbit polyclonal anti-eNOS antibody with peroxidase-linked anti-rabbit IgG for chemoluminescence and densitometry. Data are given as percentage of control absorbance units from densitometry.

Sterol Synthesis in Endothelial CELLS

Cholesterol synthesis is assayed in human arterial endothelial cells (Parker et al. 1990) grown to confluence and incubated for 16 h in serum-free standard medium + 1.0% serum albumin. Statins are added in various concentrations in DMSO vehicle and 0.5 h sterol synthesis is assayed by addition of $\text{Na-[1-}^{14}\text{C]}$ acetate ($4.0 \mu\text{Ci/well}$, 2.0 mCi/ml , 58 mCi/mmol) and incubation for 4 h at 37°C . Radiolipids in washed and lysed cells are extracted into chloroform–methanol and cell cholesterol is resolved by silica gel thin-layer chromatography. Radioactivity is determined by phosphorimager, and the 50% inhibitory concentration (IC_{50}) calculated and compared to that in vehicle controls.

EVALUATION

Data obtained by these methods suggest that the action of lipophilic statins in endothelium can shift e-NOS localization towards intracellular domains, thereby increasing the encounter with metabolically generated superoxide anion to produce peroxynitrite and related oxidants. Under some conditions, the direct action of lipophilic HMG-CoA reductase inhibitors may unbalance NO and superoxide anion fluxes and promote oxidant stress, compromising the beneficial vascular effects of e-NOS.

MODIFICATIONS OF THE METHOD

Kano et al. (1999) measured upregulation of eNOS mRNA in the rabbit aorta by a HMG-CoA reductase inhibitor using competitive reverse-transcriptase polymerase chain reaction methods.

Laufs et al. (2002) determined the expression and activity of eNOS by reverse-transcriptase polymerase chain reaction, Western blotting and arginine-citrulline assays.

Yamamoto et al. (2003) investigated the effect of a HMG-CoA reductase inhibitor on inducible nitric

oxide synthase expression in rat vascular smooth muscle cells using a modification of the semi-quantitative reverse transcription-polymerase chain reaction.

Ikeda et al. (2003) reported the protection of ischemic-reperfused myocardium in normocholesterolemic rats by a HMG-CoA reductase inhibitor.

Baetta et al. (2002) found that in the absence of lipid lowering, tissue factor expression in infiltrated inflammatory cells and macrophage accumulation in carotid lesions of cholesterol-fed rabbits are reduced by statin treatment.

Shimizu et al. (2003) performed heterotopic murine cardiac transplants in total allogeneic or histocompatibility class II-mismatched combinations. Direct anti-inflammatory mechanisms of statins may contribute to the attenuation of experimental allograft arteriosclerosis.

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M.3.3

Inhibition of Squalene Synthase

PURPOSE AND RATIONALE

Squalene synthase (farnesyl-diphosphate:farnesyl diphosphate farnesyl-transferase) is an enzyme vital for cholesterol biosynthesis. It catalyzes the dimerization of two farnesyl pyrophosphate molecules to form squalene, a key cholesterol precursor. Unlike HMG-CoA reductase inhibitors, squalene synthase inhibitors do not lower the levels of ubiquinone and dolicol *in vivo*, both essential for cell growth and viability. Several natural and synthetic squalene synthase inhibitors have been described (Biller et al. 1991a, 1991b; Oehlschlager et al. 1991; Baxter et al. 1992; Ciosek et al. 1993; Harris et al. 1995; Lindsey and Harwood 1995; Chan et al. 1996; Dufresne et al. 1996; McTaggart et al. 1996; Amin et al. 1996, 1997; Sliskovic and Picard 1997; Rosenberg 1998; Vaidya et al. 1998; Hiyoshi et al. 2000, 2003; Ugawa et al. 2000, 2002, 2003; Amano et al. 2003; Ishihara

et al. 2003; Nishimoto et al. 2003). Ugawa et al. (2000) determined squalene synthase activities in microsomes.

PROCEDURE

Microsomes are prepared from the livers of rats, hamsters, guinea pigs, beagle dogs and rhesus monkeys as well as from HepG2 cells. The tissues or harvested cells are homogenized in 50 mM HEPES buffer using a glass homogenizer. Homogenates are centrifuged at 500 *g* for 15 min at 4°C and the resulting supernatants are further centrifuged at 8000 *g* for 15 min at 4°C. Microsomes are then isolated from this second supernatant by ultra-centrifugation at 100,000 *g* for 60 min at 4°C. The microsome precipitates are suspended in HEPES buffer (1–5 mg/ml) and stored in aliquots at –80°C.

Squalene synthase activities of these microsomes are assayed using a modification of the technique of Amin et al. (1992). The test compounds are dissolved in DMSO and the assays carried out in 50 mM HEPES buffer, pH 7.5, containing (in mM): NaF 11, MgCl₂ 5.5, DTT 3, NADPH 1, farnesyl pyrophosphate (FPP) 5 μM, [³H]-FPP (0.017 μM, 15 Ci/mmol), NB-598, a squalene oxidase inhibitor, 10 μM and sodium pyrophosphate decahydrate 1 mM. After preincubation of these components at 30°C for 5 min, the reaction is started by the addition of microsomes (10 μg protein). The reaction is carried out at 30°C for 20 min, then terminated by addition of 100 μl of 1:1 solution of 40% (w/v) KOH:ethanol. Synthesized [³H]squalene is extracted in petroleum ether and counted in Aquasol-2 using a Beckman liquid scintillation counter.

EVALUATION

Results are expressed as mean ±SEM and compared using two-way repeated analysis of variance (ANOVA).

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M.3.4

Inhibition of Squalene Epoxidase

PURPOSE AND RATIONALE

Squalene epoxidase is a microsomal enzyme that catalyses the oxidation of squalene to 2,3-oxidosqualene, the last reaction of non-sterol metabolites in the cholesterol biosynthesis pathway (Hidaka et al. 1990). Inhibitors of this enzyme are unlikely to block the production of biologically important non-sterol products, such as isoprenyl adenine, dolichol, coenzyme Q, or heme A, in contrast with HMG-CoA reductase inhibitors, which inhibit their production (Ghirlanda et al. 1993; Chugh et al. 2003). Several squalene epoxidase inhibitors have been described (Horie et al. 1990, 1991; Hikada et al. 1991; Moore et al. 1992; Gotteland et al. 1998; Sawada et al. 2001, 2004).

Sawada et al. (2001) described a modified squalene epoxidase assay.

PROCEDURE

HepG2 cells are cultured routinely in medium A (Eagle's modified minimum essential medium supplemented with 1 mM pyruvate and non-essential amino acids with 10% heat-inactivated fetal calf serum) at 37°C in a humidified 95% O₂/5% CO₂ air atmosphere.

For the assay, the cells are grown in 225-cm² culture flasks, and incubated for 18 h in medium A containing 10% human lipoprotein-deficient serum and 1 μM of the squalene synthase inhibitor L-654,949, to increase the squalene epoxidase activity. The HepG2

cells are washed and harvested by trypsin treatment. After centrifugation (100 g, 5 min at 4°C), the supernatant fraction is removed by aspiration. The cell pellet is frozen and kept at –80°C until use. On the day of the experiment, the cell pellet is thawed, ruptured by sonication (5 s at 4°C) in 0.1 M Tris-HCl, pH 7.5 containing 1 mM EDTA, mixed 4:1 with 2% Triton X-100, allowed to stand at 4°C for 30 min, and assayed for squalene epoxidase activity (Tai and Bloch 1972). Aliquots of the mixture are incubated for 90 min at 37°C with or without test compound dissolved in 1% DMSO in a final volume of 0.3 ml containing 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM NADPH, 1 mM FAD, 0.3 mM AMO1618 (an inhibitor of 2,3-oxidosqualene cyclase), 0.17% Triton X-100, and 8 μM [³H]-squalene (3.7 kBq) dispersed in 0.075% Tween 80. The reaction is stopped by the addition of 0.3 ml of 10% ethanolic KOH. After incubation for 90 min at 75°C, non-saponifiable materials are extracted with 2 ml of petroleum ether. The extracts are evaporated under a nitrogen stream. The residue is taken up in a small volume of diethyl ether, spotted on a silica gel thin layer chromatography plate and developed in benzene/ethyl acetate (99.5:0.5, v/v). The radioactivity corresponding to 2,3-oxidosqualene is counted by autoradiography.

EVALUATION

The results are expressed as means ±SD and evaluated by repeated-measures analysis of variance, followed by Dunnett's multiple comparisons.

MODIFICATIONS OF THE METHOD

A simplified squalene epoxidase assay based on HPCL separation and time-dependent UV/visible determination of squalene is published by Grieverson et al. (1997).

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M.4 Inhibition of Cholesterol Absorption

M.4.1 Inhibition of ACAT (Acyl Coenzyme A: Cholesterol Acyltransferase)

M.4.1.1 General Considerations

Acyl coenzyme A:cholesterol acyltransferase (ACAT), which catalyses the intracellular formation of cholesteryl esters, plays an important role in the intestinal absorption of cholesterol, foam cell formation within the arterial wall and VLDL production in the liver. Two isoforms of the enzyme exist, ACAT1 expressed in macrophages and ACAT2 expressed in intestine and liver. Cholesterol is absorbed from the gut exclusively in the unesterified form, but appears in the lymph esterified with various long-chain unsaturated fatty acids. The enzyme responsible is ACAT, a microsomal enzyme, that utilizes long-chain fatty acyl coenzyme A and cholesterol as substrates. ACAT inhibitors also have potential actions beyond inhibition of cholesterol absorption. Inhibition of hepatic ACAT could reduce the production of cholesteryl esters for packaging into lipoproteins, while inhibition of ACAT1 in macrophages could reduce the deposition

of cholesteryl esters and prevent the formation of foam cells and atherosclerotic lesions. Nevertheless, from investigations in ACAT2 knockout mice it is known that ACAT2 is not the only rate-limiting pathway for cholesterol absorption. On normal chow these mice absorb cholesterol as efficiently as wild-type mice, only if mice were fed a cholesterol-enriched diet. ACAT2 is important for cholesterol absorption (Lee et al. 2004; Repa et al. 2004). Under physiological conditions alternative pathways compensate for the ACAT2 deficiency in mice (Hussain et al. 2005).

Human ACAT cDNA was cloned from a human macrophage cDNA library and expressed in an ACAT deficient line of Chinese hamster ovary cells. The cDNA, labelled K1, encoded an integral membrane protein of 550 amino acids (Chang et al. 1993). The inhibition of ACAT as treatment for hypercholesterolemia and atherosclerosis is an attractive target.

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M.4.1.2

In Vitro ACAT Inhibitory Activity

PURPOSE AND RATIONALE

In vitro ACAT inhibitory activity can be determined in microsomal preparations from liver or intestine of rabbits.

PROCEDURE

Hepatic or intestinal microsomes are prepared from rabbits. Prior to sacrifice, the animals receive chow supplemented with 2% cholesterol and 10% safflower oil for 6 weeks. Each assay contains 0.2 mg of microsomal protein and fatty acid-poor bovine serum albumin (3 mg/ml) in 0.04 M KH₂PO₄ buffer, pH 7.4, containing 0.05 M KCl, 0.03 M EDTA, and 0.3 M sucrose. Drug dilutions are made in DMSO (5 µl DMSO/200 µl total incubation volume). The reaction is started by the addition of [¹⁴C]oleyl CoA (50 µM, 7 dpm/pmol). After 3 min the reaction is stopped by the addition of chloroform-methanol 2:1. [³H]cholesteryl oleate is used as an internal standard. Lipid extracts are dissolved in chloroform, spotted on TLC plates (silica gel G) and developed in hexane-petroleum ether-acetic acid 80:20:1. Unlabeled, carrier cholesterol oleate is

added to the internal standard to aid band visualization with iodine vapor. The band corresponding to cholesteryl esters is then scraped into scintillation vials and radioactivity is determined by liquid scintillation spectroscopy.

EVALUATION

For each compound four concentrations are evaluated in duplicate. IC₅₀ values are determined by performing a nonlinear least-squares fit of the data to a log dose-response curve.

MODIFICATIONS OF THE METHOD

Rothblatt et al. (1977) studied ACAT activity in Fu5 rat hepatoma cells under the influence of hyperlipemic serum lipoproteins.

Mathur et al. (1981) studied ACAT activity in hepatic microsomes of cynomolgus monkeys during diet-induced hypercholesterolemia.

Einarsson et al. (1989) studied acyl-CoA: cholesterol acyltransferase activity in human liver microsomes.

Largis et al. (1989) found CL 277,082 to be a potent inhibitor of ACAT in microsomes from a variety of tissues and smooth muscle cells in culture.

Heffron et al. (1990) studied ACAT inhibition in microsomal fractions of the transformed mouse macrophage J774.

Bell et al. (1992) measured ACAT inhibition in cultured Fu5AH cells.

Field et al. (1991), Krause et al. (1993) tested the inhibition of ACAT in CaCo-2 cells.

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M.4.1.3

In Vivo Tests for ACAT Inhibitory Activity

PURPOSE AND RATIONALE

Most authors test the *in vivo* anti-atherosclerotic and antihyperlipemic effect of ACAT inhibitors in cholesterol-fed hypercholesterolemic animals (Balasubramaniam et al. 1990; Harris et al. 1992; Tanaka et al. 1944). Krause et al. (1993a) used the Zilversmit dual isotope technique (Zilversmit 1972; Cayen and Dvornik 1979) for determining cholesterol absorption in rats.

PROCEDURE

Male Sprague-Dawley rats weighing 200–225 g are fed with a diet containing 5.5% peanut oil, 0.5% cholic acid and 1.5% cholesterol with or without (controls) drugs for 1 week. On the last day, food is removed at 8:00 A.M. and the isotopes are administered beginning at 2:00 P.M. [³H]cholesterol (13 μ Ci/rat) is given by oral gavage and [¹⁴C]cholesterol (1.5 μ Ci/rat) is given by tail vein injection. The [³H]cholesterol is prepared as an emulsion by dissolving 125 mg cholesterol in 1625 mg olive oil. The oil phase is suspended by sonication in 25 ml of water containing 156 mg taurocholate (sodium salt). Each animal receives 1 ml. The intravenous dose is prepared by drying the labeled cholesterol (50 μ Ci), and then adding 300 μ l warm

ethanol followed by 12.5 ml of saline. Each animal receives 0.5 ml of this colloidal suspension. The rats are allowed to consume their respective diets at 3:00 P.M., and are sacrificed 48 h after the isotope administration.

EVALUATION

The percentage of an oral dose of cholesterol absorbed is calculated from the plasma isotope ratio (% of the oral dose in 2 ml plasma/of the intravenous dose in 2 ml plasma \times 100).

FURTHER IN VIVO METHODS

Heider et al. (1983) measured cholesterol absorption via the Zilversmit dual isotope method in rabbits.

Gillies et al. (1990) studied the regulation of ACAT activity by a cholesterol substrate pool during the progression and regression phases of atherosclerosis in rabbits with dietary induced atherosclerosis.

Krause et al. (1993b) compared the activities of two ACAT-inhibitors in normocholesterolemic and hypercholesterolemic rats, rabbits, guinea pigs and dogs.

Nagata et al. (1995) tested a new ACAT inhibitor in diet-induced atherosclerosis formation in female C57BL/6J mice.

Bocan et al. (1993) described a reduction of VLDL and vessel wall cholesteryl ester content in Yucatan micropigs after treatment with an ACAT inhibitor.

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M.4.1.4

Lymph Fistula Model for Cholesterol Absorption

PURPOSE AND RATIONALE

Direct evidence for an inhibitory effect on cholesterol absorption can be obtained by the lymph-fistula model in rats. This model also provides an indication as to the duration of inhibition and the relative selectivity of the compound on the absorption of cholesterol versus triglyceride and phospholipid.

PROCEDURE

Rats are anesthetized by an intramuscular injection of tiletamine/zolazepam (Telazol, 40 mg/kg). Silicon rubber cannulae are placed into the main mesenteric lymph duct and into the duodenum and secured with sutures. Animals are allowed to recover from surgery overnight in restraining cages while infused intraduodenally with 2% dextrose in saline containing 0.03% KCl (2.5 ml/h). Drinking water is allowed at libitum during this recovery period.

At 6:00 A.M. the following day, the drinking water is removed and a 2-h basal lymph sample is collected. Then, the animals are given the ACAT inhibitor at a specified dose as a single bolus into the duodenal cannula using a aqueous CMC/Tween suspension vehicle. Controls receive a bolus injection of the vehicle alone. Immediately after the drug dose, a lipid emulsion containing 0.1% cholesterol, 0.11% sodium taurocholate, 15% Intralipid (20%, Kabivitrium Inc.), 2.4% safflower oil, and 82.6% saline is infused into the duodenal cannula (3 ml/h). Then, four 2-h lymph collections are obtained. The lymph samples are extracted

into hexane in the presence of a stigmasterol internal standard. Total and free cholesterol are quantitated by liquid gas chromatography.

EVALUATION

Esterified cholesterol of lymph is determined from difference between total and free cholesterol.

MODIFICATIONS OF THE METHOD

The lymph fistula model can also be used to examine the effect of ACAT inhibitors on the absorption of endogenous (i. e. biliary) cholesterol. Cannulated rats are infused intraduodenally with the saline/dextrose solution to which 2% whole rat bile is added containing [¹⁴C]cholesterol. No nonradiolabeled lipid other than that in bile is infused into the animals, and hence, lymph cholesterol is exclusively of biliary origin. Hourly collections of lymph are obtained with the use of fraction collectors. Lymph is extracted with 3 volumes of ethylacetate-acetone 2:1 (Slayback et al. 1977). The total ¹⁴C label of an aliquot of lymph extract is determined by liquid scintillation spectroscopy. The lymph extracts are resuspended in 100 μl chloroform-methanol 2:1 and spotted on Whatman LK6D TCL plates. The plates are developed in hexane-diethylether-acetic acid 85:15:1. The distribution of ¹⁴C label between the cholesteryl ester and cholesterol bands are visualized and quantified by exposing the plates to phosphor imaging plates for 16 h and then scanning the imaging screens on a Molecular Dynamics Phosphorimager.

Clark and Tercyak (1984) studied the absorption of cholesterol during inhibition of mucosal acyl-CoA:cholesterol acyltransferase in mesenteric lymph fistula rats with normal pancreatic function.

Åkerlund and Björkhem (1990), Björkhem et al. (1993) used the lymph-fistula model in rats for studies on the link between HMG-CoA reductase and cholesterol 7 α -hydroxylase. The thoracic lymph duct was cannulated just proximal to the cisterna magna through an abdominal approach. The proximal part of the lymph duct was ligated. The cannula exited at the back of the animal. The lymph was allowed to flow freely from the animal to the bottom of the metabolic cages. Under these conditions, the rats could move freely in the cages during the lymphatic drainage.

CRITICAL ASSESSMENT OF THE METHOD

Gallo et al. (1987) found normal cholesterol absorption in rats in which intestinal acyl coenzyme A:cholesterol acyltransferase activity was significantly reduced by

ACAT-inhibitors. This challenges the value of ACAT-inhibition at least in normal individuals.

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M.5

Interruption of Bile Acid Recirculation

M.5.0.1

Cholestyramine Binding

PURPOSE AND RATIONALE

Cholesterol is metabolized in the liver by oxidation to bile acids which undergo enterohepatic circulation. In the untreated state, approximately 95% of the bile acids that are secreted are reabsorbed and returned to the liver, while the small loss is replaced by de novo biosynthesis from cholesterol. Increased excretion of bile acids with the feces increases the rate of oxidation of cholesterol in the liver leading to a partial depletion

of the hepatic cholesterol pool. A compensatory increase in uptake via the LDL receptors results in lower serum LDL levels. This can be achieved by addition of a bile acid binding resin, e. g., cholestyramine, to the food. The binding of unconjugated and conjugated bile-salt anions can be tested *in vitro* (Johns and Bates 1969).

PROCEDURE

Rabbits weighing 2.5–3 kg are switched from standard food to a diet containing 10–20% polymeric basic-anion exchanging resin, e. g. cholestyramine. Cholesterol levels in serum are measured at the beginning and at the end of a 4 weeks feeding period.

EVALUATION

Cholesterol levels as means \pm SD are calculated for controls and treated animals and compared by statistical analysis.

MODIFICATION OF THE METHOD

Tennent et al. (1960) tested polymeric organic bases for action on blood cholesterol in 4-day experiments and in experiments of 7–8 weeks duration in cholesterol-fed White Leghorn cockerels. The birds were given a diet containing 2% cholesterol and 5% cotton-seed oil with or without addition of polymeric bases. The increase of cholesterol and the incidence of aortic atheromatosis was decreased by polymeric organic bases.

Day (1990) compared the hypocholesterolemic activities of the bile acid sequestrants cholestyramine and cholestipol hydrochloride in cholesterol fed sea quail.

Quaternary ammonium conjugates of bile acid inhibited cholic acid binding and transport in everted ileal sacs of guinea pigs *in vitro* (Fears et al. 1990).

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M.6 Inhibition of Lipid Oxidation

M.6.0.1

General Considerations

Oxidative modification of the low density lipoproteins (LDL) has been shown to cause accelerated degradation of LDL via the scavenger receptor pathway. Under conditions of high serum LDL levels, LDL particles can migrate into the subendothelial space where oxidation of LDL can occur. The actual oxidation process is believed to begin with lipid peroxidation, followed by fragmentation to result in short-chain aldehydes. These aldehydes can form adducts with the lysine residues of apo B, creating a new epitope which is recognized by the scavenger receptor of macrophages.

During the same process, lecithin is converted to lysolecithin, which is a selective chemotactic agent for monocytes. The monocytes adhere to the arterial wall and penetrate through to the subendothelium. Once there, the monocyte changes to a tissue macrophage which takes up the oxidized LDL via the scavenger receptor. The uptake of oxidized LDL continues until the macrophage is engorged with cholesteryl esters ultimately forming a foam cell. Groups of these foam cells constitute a fatty streak. By inhibiting the oxidation of LDL, it is hoped that the modification of apo B and the production of chemotactic lysolecithin can be prevented.

The family of receptors for mammalian low-density proteins has been reviewed by Hussain et al. (1999).

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M.6.0.2

Inhibition of Lipid Peroxidation of Isolated Plasma Low-Density Lipoproteins

PURPOSE AND RATIONALE

Hypercholesterolemic Watanabe rabbits are considered to be a suitable model to study the effect of antioxidants as anti-atherosclerotic agents (Carew et al. 1987; Kita et al. 1987; Steinberg et al. 1988; Dresel et al. 1990). Plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits is used to test the inhibition of Cu²⁺-induced lipid peroxidation of isolated low density lipoproteins (LDL).

PROCEDURE

Animals of a modified Watanabe heritable hyperlipidemic rabbit strain (Gallagher et al. 1988) are used. The animals are fed over a period of 12 weeks with Purina rabbit chow diet with or without 1% of test compound or standard (probucol). Plasma samples are collected in Na₂EDTA (0.1% final concentration). LDL are isolated from each rabbit plasma using a sequential ultracentrifugation technique at $d = 1.019\text{--}1.063\text{ g/ml}$ (Mao et al. 1983). LDL are then dialyzed against phosphate buffered saline (PBS, 0.01 M sodium phosphate, 0.12 M NaCl, pH 7.4) at 4°C for 24 h.

For determination of LDL lipid peroxidation induced by Cu²⁺, 100 µg of each LDL sample is adjusted

to a volume of 1.5 ml with distilled water. Lipid peroxidation is initiated by addition of CuSO_4 to a final concentration of $5 \mu\text{M}$ followed by an incubation at 37°C for 3 h. The reaction is stopped by adding $100 \mu\text{l}$ of $50 \text{ mM Na}_2\text{EDTA}$. Fifty micrograms of LDL from the reaction mixture are added to 1.5 ml of 20% trichloroacetic acid and vortexed. Finally, 1.5 ml of 0.67% thiobarbituric acid (TBA) in 0.05 N NaOH is added and the mixture is incubated at 90°C for 30 min. Samples are centrifuged at 1500 rpm for 10 min. The absorbance of the supernatant fractions is determined at 532 nm to estimate the content of lipid peroxides (thiobarbituric acid-reactive substances). A standard curve (0–5 nmol) of malondialdehyde is generated using malondialdehyde bis(dimethyl acetal) as reference to determine the lipid peroxidation content in Cu^{2+} -treated LDL.

EVALUATION

The content of lipid peroxide in LDL is plotted against the drug concentration in LDL fractions. The extent of Cu^{2+} -induced peroxidation decreases with increasing drug concentrations. The effects of test compounds are compared to the standard.

MODIFICATIONS OF THE METHOD

Inhibition of iron-dependent lipid peroxidation by test compounds was measured by Braugher et al. (1987), Yoshioka et al. (1989).

Yamamoto et al. (1986) studied the effects of probucol on lipid storage in macrophages *in vitro* in the presence of acetylated low density lipoprotein using macrophage-like cells (UE-12) established from a human histiocytic lymphoma cell line.

Barnhart et al. (1989) used LDL from human plasma to study the concentration-dependent antioxidant activity of probucol.

Parthasarathy et al. (1986) incubated LDL from human plasma samples with rabbit aortic endothelial cells and measured the increase in electrophoretic mobility, the increase in peroxides, and the increase in subsequent susceptibility to macrophage degradation.

Mansuy et al. (1986) studied the inhibition of lipid peroxidation induced in liver microsomes either chemically by FeSO_4 and reducing agents (cysteine or ascorbate) or enzymatically by NADPH and CCl_4 .

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M.7**Internalization of Labeled LDL into HepG2 Cells****PURPOSE AND RATIONALE**

Enhanced uptake of low density lipoproteins (LDL) via the LDL receptor into liver cells results in reduced plasma cholesterol levels. This can be tested in the cultured hepatoma cell line HepG2.

PROCEDURE

Heterozygous WHHL rabbits are treated with the test compound over a period of 4 weeks. Blood is withdrawn twice weekly before, during, and after treatment for determination of total plasma cholesterol, low density lipoprotein cholesterol, total triglycerides, and high density lipoprotein cholesterol. Lipoprotein-deficient serum is prepared by ultracentrifugation (Goldstein et al. 1983). Serum of treated and untreated animals is used for the uptake assay being prepared by centrifugation to remove the clots, followed by heat inactivation of the complement system and sterilization through a 0.45- μ m filter. In each preparation, 2 mg LDL (*d*, 1.019 to 1.050 g/ml) at 10 mg/ml are iodinated by the monochloride method to a specific activity of 300 cpm/ng (Huettinger et al. 1984).

HepG2 cells are grown in 5 cm dishes in Eagle's minimum essential medium to 60% confluence. Serum prepared from treated animals and from the same animals in the pretreatment period is added from 1% to 50% to the medium used to grow HepG2 cells. After incubation of 18 h, cells are washed and incubated in Eagle's minimum essential medium plus 2% bovine serum albumin and 8 μ g labeled LDL for 3 h. The cells are then washed and solubilized in sodium hydroxide, and the content of each dish is counted for radioactivity. An aliquot is used to determine protein content.

EVALUATION

Values are given for specific uptake, which is calculated from the difference of total uptake minus uptake measured when a 40-fold excess of unlabeled LDL is present in the incubation medium of duplicate or triplicate incubations. The effect of the test drug is demonstrated by an increase of uptake with increasing percentage of serum of treated animals containing the active drug.

MODIFICATIONS OF THE METHOD

Sprague et al. (1993) measured the inhibition of scavenger receptor-mediated modified low-density lipoprotein endocytosis in cultured bovine aortic endothelial cells.

Takano and Mowri (1990) produced a monoclonal antibody which recognizes peroxidized lipoproteins.

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M.8**Influence of Peroxisome Proliferator-Activated Receptors (PPARs) and Liver X Receptors (LXRs) on Development of Atherosclerosis****M.8.1****General Considerations**

Peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) are nuclear receptors that regulate systemic glucose and lipid metabolism. They are expressed by macrophages in which they modulate cholesterol homeostasis and inflammation. These receptors heterodimerize with retinoid X receptors (RXRs) to modulate transcription at the promoters of target genes. They act as lipid sensors and bind with micromolar affinities to ligands derived from either intracellular metabolism or dietary lipids. Endogenous ligands of PPARs include fatty acids and eicosanoids, whereas metabolites of oxidized cholesterol activate the LXRs.

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M.8.2

Influence of PPAR Activation

PURPOSE AND RATIONALE

Peroxisome proliferator-activated receptors (PPARs) play a major role in atherosclerosis (Brun et al. 1996; Pineda Torra et al. 1999; Duval et al. 2002; Tailleux et al. 2003). The PPAR family consists of three proteins: α , β/δ and γ . Experimental data suggest that PPAR- α and PPAR- γ activation decreases atherosclerosis progression not only by correcting metabolic disorders, but also through direct effects on the vascular wall. PPARs modulate the recruitment of leukocytes to endothelial cells, control the inflammatory response and lipid homeostasis of monocytes/macrophages and regulate inflammatory cytokine production by smooth muscle cells. PPAR agonists, such as fibrates and thiazolidinediones, are recommended for the treatment of atherosclerosis, diabetes and obesity (Willson et al. 2000; Etgen and Mantio 2003; Francis et al. 2003; Wang and Tafuri 2003; Ferré 2004; Kota et al. 2005). Scavenger receptors are expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors (Chinetti et al. 2000). PPAR γ is a regulator of monocyte/macrophage function (Ricote et al. 1999).

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M.8.2.1

PPAR α

PURPOSE AND RATIONALE

Several studies indicate the lipid-lowering role of PPAR α in rodents (Chinetti et al. 2001; Vosper et al. 2002). PPAR α is highly expressed in the rodent liver where activation of these receptors with fibrates induces a massive increase in peroxisomal fatty acid oxidation in hepatocytes. This provides a powerful action for the clearance of fat from the serum. Fibrates also increase the expression of the liver fatty-acid-binding protein (Lawrence et al. 2000) and the hepatocyte-secreted apolipoproteins apoAI and apoAII (Vu-Dac et al. 1995). Lee et al. (1995) demonstrated that PPAR α is responsible for clofibrate-induced fatty acid oxidation by disrupting the PPAR α gene in mice. Mice lacking apolipoprotein E are characterized by severe hypercholesterolemia. Tordjman et al. (2001) reported that PPAR α deficiency reduces insulin resistance and atherosclerosis in apoE-null mice. Fu et al. (2003) found that the PPAR α agonist ciprofibrate severely aggravates hypercholesterolemia and accelerates the development of atherosclerosis in mice lack-

ing apolipoprotein E. Species differences have to be taken in account. Lawrence et al. (2001) reported that PPAR α fails to induce peroxisome proliferation-associated genes in human cells independent of the level of receptor expression.

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M.8.2.1.1

Effect of PPAR α Agonists in Mice

PURPOSE AND RATIONALE

Duez et al. (2002) described reduction of atherosclerosis by the peroxisome proliferator-activated receptor α agonist fenofibrate in mice.

PROCEDURE

Animals

Homozygous human apoA-I transgenic (Rubin et al. 1991) and apoE-deficient C57Bl/6 mouse strains were used. Human apoA-I transgenic mice were crossed with apoE-deficient mice to generate heterozygous human apoA-I transgenic mice in a heterozygous apoE-

deficient background. These mice were further mated with homozygous apoE-deficient mice to generate human apoA-I transgenic mice in a homozygous apoE-deficient background (hapoA-I Tg \times apoE-deficient mice). Human apoA-I expression was analyzed in plasma by enzyme-linked immunosorbent assay using specific antibodies (Berthou et al. 1996). Homozygous apoE-deficient mice were identified after determination of total cholesterol levels. After weaning, apoE-deficient and hapoA-I Tg \times apoE-deficient male mice were maintained on a standard pellet rodent chow.

Ten-week-old apoE-deficient male mice were given a Western diet containing 20% fat and 0.2% cholesterol before the treatment period. Then one group was treated for 8 weeks with fenofibrate (0.05% added to the diet, corresponding to 100 mg/kg per day), the other group served as control. At the end of the study, blood was collected under anesthesia by retro-orbital puncture and tissues collected for further analysis.

ApoE-deficient male mice on a C57Bl/6 background were treated at an age of 20 weeks with 100 mg/kg per day fenofibrate by gavage. Another group served as control. After 6 weeks, the animal were sacrificed and their aortic cholesterol and cholesteryl ester content were determined.

Ten-week-old hapoA-I Tg \times apoE-deficient male mice were given a Western diet for 2 weeks before treatment with either control or fenofibrate (0.05% added to the diet, corresponding to 100 mg/kg per day) for another 8 weeks. At the end of the study, blood and tissue samples were collected for further analysis.

Lipids, Apolipoprotein, and Lipoprotein Measurements

Plasma cholesterol and triglyceride concentrations were determined by an enzymatic assay adapted to microtiter plates using commercially available reagents. Plasma human apoA-I levels were measured by an immunoelectrophoretic assay using species-specific polyclonal antibodies (Berthou et al. 1996). Lipoprotein cholesterol profiles were obtained by fast-protein liquid chromatography. This system allows the separation of three major lipoprotein classes, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) + LDL and HDL according to their size.

Cholesterol Measurement in the Descending Aorta

After collection of the blood, the vasculature was gently perfused through the left ventricle with cold PBS and 5 mM EDTA. For collection of aorta for biochemical analysis, all branches and adipose tissue connected to the aorta were removed, and each aorta was carefully excised from the aortic root to the right renal

artery. The aortas were kept briefly on ice in PBS, and then blotted dry, weighed, minced, and extracted with chloroform/methanol (2:1) according to Folch et al. (1957). The lipid extracts were dried down, resuspended quantitatively in chloroform/methanol (2:1) and stored at -20°C until the time of assay. Total and free cholesterol levels in the aortic extracts were determined with an enzymatic fluorometric assay (Sparrow et al. 2001). The solvent was evaporated and the lipid residue was re-dissolved in 100 μl of reagent-grade ethanol. Aliquots of cholesterol and cholesteryl oleate standard solutions in chloroform/ethanol (1:1) were treated similarly. To determine free cholesterol, samples and standard were incubated for 1 h at 39°C in a total volume of 1.01 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.03% Triton X-100 and 0.9 mM sodium cholate. Cholesterol oxidase (Roche Molecular Biochemicals), peroxidase (Roche Molecular Biochemicals) and *p*-hydroxyphenylacetic acid (Aldrich) were added for an additional 1-h incubation at 37°C . The fluorescent product was measured (excitation 325 nm, emission 425 nm). For total cholesterol determinations, cholesterol esterase (Calbiochem) was included in the first incubation step, and cholesteryl oleate was used as standard. The esterified cholesterol content in each sample was calculated by subtracting the value of free cholesterol from that of total cholesterol. All values were expressed as nmol/aorta.

Analysis of Atherosclerotic Lesions in the Aorta

Hearts and ascending aortas were fixed in formaldehyde, and serial 10-mm-thick cryosections were cut from the aortic arch to the ventricles for quantitative analysis of atherosclerosis. The distance between each section was 50 μm , and the final magnification 2.5-fold. Sections were stained with Oil Red O and counterstained with hematoxylin. The atherosclerotic lesion and the Oil Red O-stained areas of each section were quantified using a computer-assisted video imaging system. To avoid morphological heterogeneity between mice, the expression of average atherosclerotic lesion areas in the region of the aortic sinus, from the appearance to the disappearance of the aortic valves, was normalized at ten equal sections.

EVALUATION

A non-parametric Mann-Whitney test was used to analyze for significant differences between the experimental groups. Analysis of variance (ANOVA) and Tukey post-hoc tests were used for analysis of the lesion area data.

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M.8.2.1.2

Effect of PPAR α and PPAR γ Agonists in Human Macrophages

PURPOSE AND RATIONALE

Lee and Evans (2002) reviewed the role of the peroxisome proliferator-activated receptor- γ in macrophage lipid homeostasis.

Chinetti et al. (1998) reported that PPAR α and PPAR γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway.

PROCEDURE

Transactivation Assays of the RPR-5 Compound

Transactivation assays are performed with the RPR-5 compound in A10 cells (a rat smooth muscle cell line) using full-length human PPAR α and PPAR γ and the PGL3-J₃-TK vector (Vu-Dac et al. 1995). After 24 h, luciferase activity is measured with LucLite (Packard, Meridian, Conn., USA) according to the manufacturer's instructions.

Cell Culture

Mononuclear cells are isolated from the blood of healthy normolipidic donors by Ficoll gradient centrifugation and cultured (Chinetti et al. 1998). Mature monocytes are used for experiments after 10 days of culture. For treatment with the different activators, medium is changed to medium without human serum but supplemented with 1% Nitridoma

HU (Boehringer). Efflux studies are performed in the absence of any serum. Human monocytic THP-1 cells (ATCC, Rockville, Md., USA) are maintained in RPMI 1640 medium containing 10% of FCS and differentiated for 48 h with 100 nM PMA.

RNA Extraction and Analysis

After incubation with the PPAR activators, cells are washed with PBS and used for RNA extraction. Total RNA is extracted after 10 days from differentiated macrophages and THP-1 cells treated for 24 h with the compounds using Trizol (Life Technologies, France). For Northern blot analysis, membranes are hydrolyzed containing 10 µg of total RNA with radiolabeled ABCA1 or 36B4 control cDNA probes. An *EcoRI* LXR- β and a *HindIII-HincII* LXR- α cDNA fragment are used as probes. For the human ABCA1 probe, a 1.1-kb cDNA fragment is cloned into a pCR4-TOPO vector (Invitrogen).

Transient Transfection Assay

At 24 h before transduction, COS1 cells are plated in 24-well plates at a density of 5×10^4 cells/well and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells are transfected by lipofection using ExGen 500 (Euromedex) in OPITMEM 1 medium, with 50 ng of reporter plasmid and increasing quantities of LXR- α and RXR expression plasmids (10, 25, and 50 ng) in the presence of 50 ng of the internal control β -gal expression vector, for 3 h in serum-free medium. Fresh medium containing 0.2% fetal calf serum and 10 µM 22(R)-hydroxycholesterol (Sigma) or its solvents are subsequently added and cells are further incubated for 48 h.

Cholesterol Loading and Efflux

Ten day-old human macrophages were pretreated for 24 h and thereafter every 24 h with PPAR activators (e. g., rosiglitazone 100 nM, Wy14643 50 µM) and cholesterol loaded by incubation with AcLDL (50 µg/ml, with or without [3 H]cholesterol) in RPMI 1640 medium supplemented with 1% Nutridoma (Boehringer) for 48 h. After this incubation period, cells are washed twice in PBS and apoAI-mediated cholesterol efflux studies are immediately performed by adding fresh RPMI medium without Nutridoma with or without 100 µg/ml of apoAI for 24 h. Because, in macrophages, the equilibrium between esterified and free cholesterol is not obtained even after a 24-h additional incubation period, the experiments are performed in the absence of equilibrium.

EVALUATION

For transactivation assays

ED₅₀ values are calculated after curve fitting with XLFit (human PPAR α EC₅₀ = 0.13 µM, PPAR γ EC₅₀ = 5 µM).

For transient transfection assay

Cell extracts are prepared and assayed for luciferase activity (Delerive et al. 2000). Results are normalized to internal control β -gal activity.

For cholesterol binding and efflux

At the end of the incubation, intracellular lipids are extracted in hexane/isopropanol, dried under nitrogen, and free cholesterol, total cholesterol and phospholipids are measured by enzymatic assays (Boehringer). Esterified cholesterol is measured as the difference between total and free cholesterol. Cellular proteins are collected by digestion in NaOH and measured by Bradford assay (BioRad). The percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free medium is expressed according to the following equation:

$$\begin{aligned} & \text{Percent decrease in cellular cholesterol} \\ &= \left\{ \left[(\text{cellular cholesterol})_{\text{RPMI}} \right. \right. \\ & \quad \left. \left. - (\text{cellular cholesterol})_{\text{apoAI}} \right] \right. \\ & \quad \left. + (\text{cellular cholesterol})_{\text{RPMI}} \right\} \times 100. \end{aligned}$$

In the experiments with [3 H]cholesterol, radioactivity is measured, by scintillation counting, in centrifuged medium and in cellular lipids extracted with hexane/isopropanol. ApoAI-induced [3 H]cholesterol is measured as the fraction of total labeled cholesterol appearing in the medium in the presence of apoAI after subtraction of values for apoAI-free medium. For the phospholipid efflux study, lipids are isolated from culture medium by chloroform/methanol extraction. Extracted lipids are subsequently dried under nitrogen pressure and phospholipid mass determined by an enzymatic assay (Boehringer).

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M.8.2.2

PPAR γ

PURPOSE AND RATIONALE

PPAR γ is a transcription factor required for the activation of many adipose-specific genes (Vosper et al. 2002). PPAR γ essentially controls the accumulation of lipids in adipocytes and many experiments have demonstrated the ability of the receptor to direct adipocyte stimulation (Rosen et al. 2000). Reginato et al. (1998) reported that prostaglandins can both promote and block adipogenesis through opposing effects on PPAR γ . PPAR γ is a molecular target for the thiazolidine group of insulin-sensitizing drugs (Rieusset et al. 1999). PPAR γ promotes lipid clearance in the liver of mice (Memon et al. 2000).

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M.8.2.2.1

Effect of PPAR γ Agonists in Mice

PURPOSE AND RATIONALE

Collins et al. (2001) reported the inhibition of the formation of early atherosclerotic lesions in diabetic and nondiabetic LDL receptor-deficient mice by a PPAR γ agonist.

PROCEDURE

Transendothelial Monocyte Migration

THP-1 cells, a human monocytic leukemia cell line, were added to a human aortic EC monolayer cover-

ing a gelatin-coated 8-mm porous membrane and incubated for 30 min at 37°C to facilitate their attachment. Cells were then pretreated with the ligands or vehicle (dimethyl sulfoxide) for 30 min at 37°C. Migration was induced by the addition of monocyte chemoattractant protein-1 (MCP-1, 50 ng/ml) to the lower compartment. After 90 min, non-migrating THP-1 cells and human aortic ECs were removed with a cotton tip, and the membranes were fixed and stained with the Quik-Diff Stain Set (DADE, Miami, Fla., USA) to identify migrated cells. The number of migrated cells was determined per 3320 high-power field. Experiments were performed in duplicate and were repeated at least 3 times.

Western Blots

Western immunoblots were performed (Goetze et al. 1999). Membranes were incubated with rabbit polyclonal antibodies (1:1000 dilution, New England Biolabs) that recognize either: (1) total extracellular signal-regulated kinase (ERK) or (2) ERK phosphorylated on threonine 202 and tyrosine 204.

Animals and Diets

Male LDLR2/2 mice were obtained (C57BL/6J-Ldlrtm1Her, stock No. 002207, Jackson Laboratory) and were group-housed under a 12-h light and 12-h dark regimen. At 3 months of age, the mice were randomly assigned to one of five dietary regimens: (1) chow (Harlan Teklad 8604), (2) high-fat complex carbohydrate (Research Diets), (3) high-fat complex carbohydrate with 4 g troglitazone/kg of food, (4) high fructose (Research Diets), or (5) high fructose with 4 g troglitazone /kg of food. The high-fat diet consisted of 21% fat, 20% protein, 50% carbohydrate, and 0.15% cholesterol. The high-fructose diet contained 4% fat, 16% protein, 71% fructose, and 0.15% cholesterol. Mice and feed were weighed weekly, and the rate of consumption of drug was computed. The mice were fed for a period of 12 weeks.

Metabolic Measurements

Blood samples from the retro-orbital sinus were obtained from the mice before the beginning of treatment and every month thereafter and from the abdominal vena cava at euthanasia. Mice were fasted overnight before the collection of the blood samples. Plasma glucose was measured by glucose oxidase reaction (Beckman Glucose Analyzer 2, Beckman Instruments). Plasma lipids and plasma insulin were measured. Blood pressures were obtained by using an

indirect tail-cuff method with a controlled-temperature chamber.

Vessel Preparation and Image Analysis

Mice were euthanized and perfused with 7.5% sucrose in 4% paraformaldehyde. Aortas were dissected out, split longitudinally, pinned flat in a dissection pan, and stained with Sudan IV to detect lipids and determine lesion area. Images were captured by use of a Sony 3-CCD video camera and analyzed using ImagePro image analysis software. The extent of lesion formation is expressed as the percentage of the total aortic surface area covered by lesions.

Cross Sections: Determination of Intimal Macrophage Content

The largest lesions from the aortic arch were excised and embedded in paraffin. The avidin-biotin-peroxidase complex technique for immunostaining was used. Macrophages were stained by using monoclonal antibody to CD68 (titer 1:100, KP1 clone, M0814, Dako). Non-immune serum was used as a control. Primary antibody incubations were performed in 1% BSA/2% goat serum containing PBS for 60 min. Biotinylated rabbit anti-mouse (Dako) was applied; incubation with a streptavidin-peroxidase complex followed. Peroxidase activity was detected with the use of diaminobenzidine tetrahydrochloride as a chromogen. Slides were then counterstained with hematoxylin. Images of the stained sections were analyzed by using the software described above. After tracing the intimal area to be measured with a cursor, five pixels of color, which defined the anti-CD68 stain, were sampled by the operator. The area encompassed by the pixels, which was not contiguous, in the color range for anti-CD68 was then computed automatically by the software.

EVALUATION

Statistical analysis was performed by using 2-factorial ANOVA with Student-Newman-Keuls to determine the differences between individual group means.

MODIFICATIONS OF THE METHOD

Li et al. (2000) used LDL-receptor deficient mice to study the inhibition of atherosclerosis by PPAR γ ligands.

Chen et al. (2001) used apolipoprotein E knockout mice to study the inhibition of atherosclerosis by a thiazolidinedione.

Gavrilova et al. (2003) used lipotrophic A-ZIP/F-1 mice to study the contribution of peroxisome proliferator-

activated receptor γ to hepatic steatosis, triglyceride clearance, and regulation of body fat mass.

Tao et al. (2003) found antioxidative, antinflammatory, and vasoprotective effects of a peroxisome proliferator-activated receptor- γ agonist in hypercholesterolemic New Zealand white rabbits.

Iwaki (2003) used C57BL/KsJ (*db+/*db+**) mice to study the induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors.

Corti et al. (2004) performed an *in vivo* study by high-resolution magnetic resonance imaging of male New Zealand white rabbits. A selective PPAR γ agonist showed an additive effect on plaque regression in combination with a statin in experimental atherosclerosis.

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M.8.2.2.2**Effect of PPAR γ Agonists on Gene Expression in Macrophages****PURPOSE AND RATIONALE**

Hodgkinson and Ye (2003) used a combination of expression microarray and Northern blot analyses to identify target genes for peroxisome proliferator-activated receptor (PPAR) γ in RAW264.7 macrophages. PPAR γ natural ligand 15-deoxy- Δ 12-14 prostaglandin and synthetic ligands ciglitazone and rosiglitazone increased the expression of scavenger receptor CD36 and ATP-binding cassette transporter A1, as well as adipophilin (a lipid droplet coating protein involved in intracellular lipid storage and transport), calpain (a protease implicated in ABCA1 protein degradation), and ADAM8 (a disintegrin and metalloprotease protein involved in cell adhesion).

PROCEDURE**Cell Culture**

Macrophage RAW264.3 cells were purchased from ATCC and cultured in DMEM (Sigma, Poole, UK) at 37°C in a humidified atmosphere with 95% air-5% CO₂. When cells reached ~80% confluence, WY14,643, 15d-PGJ2, ciglitazone, rosiglitazone, and BADGE were added to the medium as described below to give final concentrations of 100, 5, 50, 1, and 500 μ M, respectively.

RNA Extraction

Total RNA from RAW264.7 macrophages was extracted using the BD Clontech Nucleobond RNA purification system (BD Clontech, Basingstoke, UK). Cells were lysed by a lysis buffer containing guanidine thiocyanate. After removing cellular proteins from the lysate, RNA was precipitated, then immobilized in a column containing silica glass fibers, and subjected to RNase-free DNase I digestion to remove genomic DNA. The RNA sample was further purified by washing and finally eluted from the column into nuclease-free water. The integrity of the RNA sample was verified by spectrophoretic analysis and formaldehyde agarose gel electrophoresis.

Microarray Filters and Hybridization

cDNA microarray nylon filters were purchased from Research Genetics. Radiolabeled cDNA probes were generated by reverse transcription of RNA extracted from rosiglitazone- or ciglitazone-treated and untreated (control) RAW264.7 macrophages using a Research Genetics GeneFilters Probe Labeling and

Purification kit (Invitrogen, Paisley, UK). Briefly, 4–10 μ g of total RNA was mixed with 0.4 mg of oligo(dT) and incubated at 70°C for 10 min. Then, 10 nmol each of dATP, dGTP, and dTTP, 37.5 U of AMV reverse transcriptase, and 100 μ Ci of [α -³³P]dCTP (3000 Ci/mmol; Amersham, Bucks, UK) were added, and the solution (30 μ l of final volume) incubated at 42°C for 2 h. The labeled cDNA probe was then purified using spin columns supplied with the kit to remove unincorporated radioactive nucleotide. Prehybridization was carried out in 5 ml of MicroHyb buffer (Research Genetics) supplemented with poly(dA) (1 μ g/ml) and denatured human Cot-1 DNA (1 μ g/ml) at 42°C for 2 h. Radiolabeled cDNA probe was denatured at 100°C for 3 min and then added to the above solution, followed by hybridization at 42°C overnight. The filters were then washed twice in a solution containing 2 \times SSC and 1% SDS at 50°C for 20 min and a brief wash (5 min) in a solution containing 0.5 \times SSC and 1% SDS at room temperature. The filters were exposed to a low-energy phosphor storage screen, which was then scanned using a Storm phosphorimager (Molecular Dynamics), and the resultant TIFF images analyzed using Pathways 4 software (Research Genetics). The intensities of the spots were normalized to mean intensities of all spots on each filter. Normalized intensities of corresponding spots were compared between two filters. In order to check the data, the filters were stripped and re-probed with the opposite radiolabeled cDNA probe, thus a filter probed with control cDNA was stripped and probed with treated cDNA and vice versa.

Northern Blot and Hybridization

RNA was denatured in 50% (v/v) formamide and 6% (w/v) formaldehyde at 65°C for 10 min and subjected to electrophoresis on 1.5% (w/v) agarose gel in MOPS buffer [20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0)] containing 1.9% (w/v) formaldehyde. Integrity and equal loading of the RNA was confirmed by observing ethidium-bromide-stained 28S and 18S mRNA bands under UV illumination. The RNA was transferred overnight to Hybond-N nylon membrane (Amersham) by capillary action and the RNA linked to the membrane by baking at 80°C for 2 h. cDNA probes for ABCA1, ADAM8, adipophilin, calpain-L3, CD36, protein phosphatase 2A non-catalytic subunit, and thymosin were generated from the IMAGE clone which had been isolated, sequenced, and verified by Research Genetics. Purified plasmid DNA was cut with the appropriate restriction enzymes and the digested insert was purified by

electrophoresis on 0.8% agarose in TAE buffer [4 mM Tris, 1 mM EDTA, and 0.0114% (v/v) acetic acid (pH 7.6)] containing 1 µg/ml ethidium bromide. The purified insert was purified from the gel using a Qiagen Gel Extraction kit (Qiagen, Crawley, UK) and 25 ng of cDNA was denatured at 95°C for 2 min and the cDNA was radiolabeled with [α -³²P]dCTP by random priming using the Klenow Labeling kit as supplied by Promega. Unincorporated label from cDNA labeling was removed using a QIAquick Nucleotide Removal kit (Qiagen). Hybridizations with cDNA probes were performed with PerfectHyb (Sigma, Poole, UK) at 65°C. Prehybridization was carried in 5 ml PerfectHyb supplemented with 150 µg of denatured salmon sperm DNA (Sigma) for 2 h. Hybridization with the radiolabeled cDNA probe was carried out in the same mixture overnight. Membranes were washed twice at room temperature with 2 × SSC–0.1% SDS for 10 min followed by a 10 min wash in 0.1 × SSC–0.1% SDS. A high-stringency wash (5–10 min) was performed in 0.1 × SSC–0.1% SDS at 65°C. The membranes were then subjected to autoradiography at –70°C for between 12 h and 7 days.

EVALUATION

Autoradiographs of Northern blots were scanned and analyzed using Phoretix software.

MODIFICATIONS OF THE METHOD

Tontonoz et al. (1988) reported that PPAR γ promotes the differentiation of monocytes/macrophages and the uptake of oxidized LDL.

Moore et al. (2001) reviewed the role of PPAR γ in macrophage differentiation and cholesterol uptake.

Arimura et al. (2004) found that the peroxisome proliferator-activated receptor γ regulates expression of the perilipin gene in adipocytes. Perilipins are a family of proteins coating the surface of lipid droplets in adipocytes.

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M.8.2.3 PPAR δ

PURPOSE AND RATIONALE

Vosper et al. (2001) reported that PPAR δ promotes lipid accumulation in human macrophages.

PROCEDURE

Isolation of Human Monocytes

and In Vitro Monocyte/Macrophage Differentiation

Human monocytes were isolated from buffy coat preparations of whole blood taken from healthy volunteers. The buffy coat was mixed with OptiprepTM (Robbins Scientific) in a ratio of 2.5:1 and then overlaid with a discontinuous OptiprepTM gradient. Following centrifugation for 25 min at 600 g the monocyte layer formed within the top 5–10 ml of the gradient was removed, washed with phosphate-buffered saline, and resuspended in RPMI 1640, supplemented with 2 mM glutamine and 10% human serum (Sigma). Cell viability was assessed by the ability to exclude trypan blue and was typically 95%. Monocyte purity was determined by differential counts of DiffQuik-stained (Porvair Sciences) cell preparations and was typically >95%. For monocyte-macrophage differentiation, monocytes isolated as above were resuspended in culture medium at a density of 2.5 × 10⁶/ml and seeded into 12-well tissue culture plates; medium was changed every 48 h.

Transient Transfection

COS-1 cells were transfected by the DEAE-dextran method. The reporter construct pFABPLUC contained four copies of the peroxisome proliferator response element from the human liver FABP gene in front of the herpes simplex virus-thymidine kinase promoter, cloned immediately upstream of the cDNA encoding firefly luciferase in pGLBAS (Promega). The PPAR expression vectors contained the coding sequence for human PPAR α , δ , and γ under the control of the enhancer/promoter of the human cytomegalovirus. PSV-Gal was co-transfected with each sample to act as an internal control for transfection. Cell lysates were prepared and luciferase and β -galactosidase activities were assayed using kits as described by the manufacturer (Promega).

Data are presented as the relative light units obtained with the luciferase assay divided by the ab-

sorbance obtained at 405 nm in the β -galactosidase assays (Luc/ β -galactosidase).

Culture and Differentiation of THP-1 Cells

THP-1 cells were obtained from ATCC. Cultures were grown in RPMI supplemented with 10% heat-inactivated fetal calf serum. Differentiation was initiated by the addition of 5 ng/ml PMA in the above medium. All drugs were added in Me₂SO and were replaced at intervals of 48 h. After 4 days the cells were fixed with 0.66% paraformaldehyde then stained with Oil red O and hematoxylin. Quantification of lipid accumulation was achieved by extracting Oil red O from stained cells with isopropyl alcohol and measuring the optical density of the extract at 510 nm. The value obtained using a control culture was subtracted from the resulting values. The Oil red O absorbance was corrected by co-staining DNA with SYBR green dye (Molecular Probes) and quantified on a Labsystems FluoroSkan Ascent FL microplate fluorimeter. Cell number was determined from a standard curve.

Isolation of Stable Cell Lines

The entire coding sequence of human PPAR was subcloned, in both sense and antisense orientations, into the eukaryotic expression vector pCLDN. The resulting plasmids were transfected into THP-1 cells using a modified DEAE-dextran procedure. The cells were maintained in medium containing 1 mg/ml G418 and 10% THP-1 conditioned medium, with rigorous washing procedures to remove dead cells. This was continued until cell killing stopped and robust growth was observed.

Western Blotting

Cultures were lysed in SDS-polyacrylamide gel electrophoresis loading buffer and analyzed by Western blotting using standard procedures. PPAR antiserum was used at a 1:2000 dilution and the anti-AFAR antiserum was used at a 1:3000 dilution. A peroxidase-conjugated mouse anti-rabbit IgG antiserum (Sigma) was used as a secondary detection reagent at 1:3000 and the results were visualized using enhanced chemiluminescence (ECL+) as described by the manufacturer (Amersham Pharmacia Biotech).

Lipid Extraction and Measurement

Cultures were treated as above and then extracted with methanol/chloroform/phosphate-buffered saline (1:1:1, v/v) containing stigmasterol as an internal standard for cholesterol. The organic phase was analyzed for cholesterol with and without saponification by

GC-MS using a Finnegan ThermoQuest Trace 2000. Triglycerides were quantified colorimetrically using a kit from Sigma and triolein as a standard. Protein concentrations were determined using the Bio-Rad Protein assay reagent.

Cholesterol Efflux Assays

THP-1 cells were differentiated with PMA in the absence or presence of 100 nM compound F for 3 days. PPAR SENSE cells were differentiated with PMA for 3 days. The cells were labeled with 2 μ Ci (40 nmol) of [4-¹⁴C]cholesterol in 2 ml of medium containing 10% fetal calf serum for 3 h. ApoA1-specific efflux was determined (Oliver et al. 2001). For apoA1-independent efflux assays the cells were incubated for a further 120 h in medium containing 10% fetal calf serum.

Radioactivity release into the medium was determined by scintillation counting at 1, 4, 18, 24, 48, 72, and 120 h. All cultures incorporated similar levels (between 80 and 95%) of the labeled cholesterol.

RNA Extraction and Analysis

RNA was extracted from THP-1 cells using TRIzol reagent as recommended by the manufacturer. cDNA was synthesized from such RNA using "You-Prime Ready-To-Go" beads from Amersham Pharmacia Biotech. TAQMANTM real time PCR analysis was applied using prepared reagents from PerkinElmer LifeSciences. The primers and probes are described elsewhere (Kliewer et al. 1992). Relative levels of mRNA were calculated using the values obtained with each target gene compared with the values obtained with the 18 S ribosomal RNA probes.

EVALUATION

All graphs and statistics were prepared using Graphpad Prism for the Macintosh v3.0 (Graphpad Inc., San Diego, Calif., USA). Non-linear regression was applied using a sigmoidal response model for the dose response to compound F in transient transfection experiments. *P*-values were calculated using a standard Student's *t*-test.

MODIFICATIONS OF THE METHOD

Wang et al. (2004) described the engineering of a mouse capable of continuous running up to twice the distance of a littermate. This was achieved by targeted expression of an activated form of PPAR δ in skeletal muscle, which induces a switch to form increased numbers of type I muscle fibers. Treatment of wild-type mice with PPAR δ agonist elicits a similar type I fiber gene expression profile in muscle.

Forman et al. (1997) developed a conformation-based assay that screens activators for their ability to bind to PPAR α/δ and induce DNA binding. Specific fatty acids, eicosanoids, and hypolipidemic drugs are ligands for PPAR α or PPAR δ . Because altered fatty levels are associated with obesity, atherosclerosis, hypertension, and diabetes, PPARs serve as molecular sensors that are central to the development and treatment of these metabolic disorders.

PROCEDURE

Cell Culture and Transfection

CV-1 cells were grown and transfected (Forman et al. 1995a). The reporter construct, PPEx3 TK-LUC, contained three copies of the acyl-CoA oxidase PPRE upstream of the Herpes virus thymidine kinase promoter (Kliwer et al. 1992). Expression vectors contained the cytomegalovirus IE promoter/enhancer (pCMX) upstream of wild-type mouse PPAR α , mouse PPAR γ 1- Δ N (Met-105-Tyr-475), mouse PPAR δ - Δ N (Leu-69-Tyr-440), mouse PPAR α -G (Glu-282 Gly) (Hsu et al. 1995), or *Escherichia coli* β -galactosidase as an internal control. Cells were exposed to the compounds for 24 h and then harvested and assayed for luciferase and β -galactosidase activity. All points were performed in triplicate and varied by less than 10%. Normalized luciferase activity was determined and plotted as *n*-fold activation relative to untreated cells. Each experiment was repeated three or more times with similar results.

Electrophoretic Mobility-Shift Assays

In vitro-translated mouse PPAR α (0.2 μ l) and human RXR α (0.1 μ l) were incubated for 30 min at room temperature with 100,000 cpm of Klenow-labeled acyl-CoA oxidase PPRE as described (Forman et al. 1995b) but with 150 mM KCl.

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M.8.3

Influence of Liver X Receptor Agonists

PURPOSE AND RATIONALE

Liver X receptors (LXR) are nuclear receptors that are intracellular sensors, which regulate the expression of genes controlling cholesterol absorption, excretion, catabolism and cellular efflux in target organs including small intestine, liver and macrophages. The LXRs belong to a subgroup of nuclear receptors that are considered "metabolic receptors". Also included in this group are the PPARs and FXR (farnesol-activated receptor). The endogenous ligands for these receptors are intermediates or endproducts of metabolic pathways. The two LXRs, LXR α and LXR β , are activated by physiological concentrations of oxidized cholesterol, such as 22(R)-hydroxycholesterol and 24(S),25-epoxycholesterol. LXR α is expressed at high levels in liver, intestine adipose tissue, and macrophages, whereas LXR β is expressed ubiquitously (Konvanen and Pentikäinen 2003; Jaye 2003; Joseph and Tontonoz 2003). Macrophage liver XC receptors are identified as inhibitors of atherosclerosis (Tangirala et al. 2002). Liver X receptors are regarded as potential therapeutic agents for dyslipidemia and atherosclerosis (Lund et al. 2003).

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M.8.3.1

Stimulation of Cholesterol Efflux

PURPOSE AND RATIONALE

LXRs induce ATP binding cassette (ABC) transporters, e. g., ABCA1 (Costet et al. 2000) and ABCG1 (Venkateswaran et al. 2000), which are involved in the transport of cholesterol and phospholipids from cells to extracellular receptors.

Sparrow et al. (2002) described a potent synthetic LXR agonist that is more effective than 22(R)-hydroxycholesterol in inducing ABCA1 mRNA and stimulating cholesterol efflux.

PROCEDURE

Cells

Cells were cultured at 37°C in a humidified atmosphere consisting of 95% air and 5% carbon dioxide. Primary human fibroblasts were obtained from the Camden human cell repository. The cells were grown, handled, and cholesterol-loaded as described by Francis et al. (1955). Human primary hepatocytes were maintained in phenol red-free Dulbecco's modified Eagle's medium (high glucose) containing 10% charcoal-stripped FCS, 1% non-essential amino acids, 1% glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate. Caco-2 cells were obtained from ATCC and grown in Opti-MEM containing 10% FCS, non-essential amino acids, and vitamins. THP-1 cells were obtained from ATCC and were grown in RPMI medium containing 10% FCS, 0.05 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotic-antimycotic solution (100 units/ml penicillin, 0.1 µg/ml streptomycin, 0.25 µg/ml amphotericin B). THP-1 cells were differentiated into macrophages in six-well tissue culture dishes at a density of 1 million cells/well by incubation in the same medium plus 100 nM tetradecanoyl phorbol acetate for 3 days. After differentiation into macrophages, cells were used for mRNA measurements or for cholesterol efflux assays as described below. Human primary monocytes were prepared as described by Wright and Silverstein (1982) and differentiated to macrophages by culturing for 7 days in Teflon jars in RPMI 1640 medium supplemented with 12% human serum, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. They were then plated in the same medium to initiate experiments.

Assays of Cholesterol Efflux Using [³H]Cholesterol

Cells were labeled by incubation for 24 h in fresh growth medium containing [³H]cholesterol (10 µCi/ml). In some experiments, cells were simultaneously labeled with [³H]cholesterol and cholesterol-loaded using acetylated LDL. Following labeling with [³H]cholesterol, cells were washed and incubated for an additional 24 h in serum-free media containing 1 mg/ml bovine serum albumin to allow for equilibration of [³H]cholesterol with intracellular cholesterol. Cholesterol efflux was initiated by adding the indicated amount of apoA-I, usually 10 µg/ml, with or without APD, in serum-free medium. APD was added to cell culture medium from Me₂SO solutions, and control cells received an equivalent amount of Me₂SO, never exceeding 0.1%. After 24 h, media were harvested, and cells were dissolved in 1 mM HEPES, pH 7.5 containing 0.5% Triton X-100. Media were briefly centrifuged to remove non-adherent cells, and then aliquots of both the supernatants and the dissolved cells were subjected to liquid scintillation spectrometry to determine radioactivity.

Assays of Cholesterol Efflux

Using Gas Chromatography-Mass Spectrometry

At the end of the 24-h efflux assay, cells and media were extracted. Cells were extracted twice (10 min with shaking each time) with 1 ml of hexane/isopropyl alcohol/water (3:2:0.1, v/v/v)/5 cm² of cell surface area. Media were extracted with 2.5 vols of chloroform/methanol (2:1, v/v). Internal standard [²H]₆cholesterol, was added to the extracts, and the samples were taken to dryness under a stream of argon. Half of the cellular lipid extract was saponified for 1 h at 60°C in a solution of 3% KOH in 90% ethanol. One volume of water was added, and the mixture was extracted with 2 vols of hexane. The extracts of media and cellular lipids with or without saponification were taken to dryness under argon, derivatized to trimethylsilyl ethers by treatment with Sigma Sil-A for 1 h at 60°C, redissolved in hexane, and analyzed by gas chromatography/mass spectrometry. The amount of cholesterol in the original sample was determined using a standard curve.

Gas Chromatography/Mass Spectrometry

Gas chromatography/mass spectrometry was performed using a ThermoQuest GCQ instrument equipped with an RTX-5MS column (30 m × 0.25 mm inner diameter, 0.25-µm phase thickness; Restek, Bellefonte, Pa., USA). The gas chromatography program was 180°C for 1 min, followed by a temperature

gradient of 20°C/min to 290°C and a final elution at 290°C for 20 min. The injector was operated in the split mode (1:10 split), and the temperature was kept at 275°C. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The instrument was operated in the electron ionization mode with the electron energy set to 70 eV. The ion trap was used for the selected ion monitoring of m/z 458 and 464 for determination of cholesterol and [26,27-²H₆]cholesterol, respectively.

Transactivation Assays

Expression constructs were prepared by inserting the ligand binding domain of human LXR α and LXR β cDNAs adjacent to the yeast GAL4 transcription factor DNA binding domain in the mammalian expression vector pcDNA3 to create pcDNA3-LXR α /GAL4 and pcDNA3-LXR β /GAL4, respectively. The GAL4-responsive reporter construct, pUAS(5x)-tk-luciferase, contained five copies of the GAL4 response element placed adjacent to the thymidine kinase minimal promoter and the luciferase reporter gene. The transfection control vector, pEGFP-N1, contained the green fluorescence protein gene under the regulation of the cytomegalovirus promoter. For transient transfections, HEK-293 cells were seeded at 4×10^4 cells/well in 96-well plates in Dulbecco's modified Eagle's medium (high glucose) containing 10% FCS, 100 units/ml penicillin G, and 100 μ g/ml streptomycin sulfate at 37°C in a humidified atmosphere of 5% CO₂. After 24 h, transfections were performed with LipofectAMINE (Invitrogen) according to the instructions of the manufacturer. Transfection mixes contained 0.002 μ g of LXR α /GAL4 or LXR β /GAL4 chimeric expression vectors, 0.02 μ g of reporter vector pUAS(5x)-tk-luc, and 0.034 μ g of pEGFP-N1 vector as an internal control of transfection efficiency. Compounds were characterized by incubation with transfected cells for 48 h across a range of concentrations in phenol red-free Dulbecco's modified Eagle's medium (high glucose) containing 10% charcoal-stripped FCS, 1% non-essential amino acids, 1% glutamine, and 100 units/ml penicillin G and 100 μ g/ml streptomycin sulfate. Cell lysates were prepared from washed cells using cell lysis buffer (Promega, Madison, Wis., USA).

Measurement of mRNA Levels by Real-Time Quantitative Reverse Transcription-PCR (TaqMan)

Real-time quantitative PCR analysis was used to determine the relative levels of ABCA1 and ABCG1 mRNA. Reverse transcription and PCRs were performed according to the manufacturer's instructions.

Sequence-specific amplification was detected with an increasing fluorescent signal of FAM (reporter dye) during the amplification cycle.

Amplification of the mRNA for the human 23-kDa highly basic protein, also called ribosomal protein L13a, was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Levels of the different mRNAs were subsequently normalized to highly basic protein mRNA levels. Oligonucleotide primers and Taq-Man probes were designed using Primer Express software (Applied Biosystems) and were synthesized by Applied Biosystems. Sequences of forward primers, reverse primers, and probes (respectively) were as follows:

ABCA1, TGTCCAGTCCAGTAATGGTTCTGT, AAACGAGATATGGTCCGGATT, 6FAM-ACACCTGGAGAGAAGCTTTCAACGAGACTAACCTAMR A; ABCG1, TGCAATCTTGTGCCATATTTGA, CCAGCCGACTGTTCTGATCA, 6FAM-TACCACAACCCAGCAGATTTTGTCTATGGA-TAMRA; SREBP-1c, GGTAGGGCCAACGGCCT, CTGTCTTGGTTGTTGATAAGCTGAA. 6FAM-ATCGCGGAGCCA TGGATTGCACT-TAMRA; HBP, GCTGGAAGT ACCAGGCAGTGA, ACCGGTAGTGGATCTTGGCTTT, VIC-TCTTTCCTTCTTCTCCTCCAGGGTGGCT-TAMRA.

EVALUATION

For Cholesterol Efflux

Cholesterol efflux is expressed as a percentage, calculated as $([^3\text{H}]\text{cholesterol in medium})/([^3\text{H}]\text{cholesterol in medium} + [^3\text{H}]\text{cholesterol in cells}) \times 100$.

For Transactivation Assay

Luciferase activity in cell extracts was determined using luciferase assay buffer in a ML3000 luminometer (Dynatech Laboratories). Green fluorescence protein expression was determined using the Tecan Spectrofluor Plus at an excitation wavelength of 485 nm and emission at 535 nm. Luciferase activity was normalized to green fluorescence protein expression to account for any variation in efficiency of transfection.

MODIFICATIONS OF THE METHOD

Chawla et al. (2003) used null stem cell of mice to demonstrate that PPAR δ is a very low-density lipoprotein sensor in macrophages.

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M.8.3.2

Liver X Receptor Binding

PURPOSE AND RATIONALE

Several authors have studied the structure and function of the liver X receptors (Spencer et al. 2001; Yoshikawa et al. 2002; Hoerer et al. 2003; Yu et al. 2003; Williams et al. 2003).

Janowski et al. (1999) studied the structural requirement of ligands for the oxysterol liver X receptors LXR α and LXR β and developed a ligand binding assay.

PROCEDURE

Ligands

Purchased ligands include the following: 9-*cis*-[20-methyl-³H]retinoic acid (72 Ci/mmol; 1 Ci = 37 GBq) and [26,27-³H(N)]-24(S),25-EC (76 Ci/mmol); 22(R,S)-HC, 25-HC, 7 α -HC, 24,25-dehydrocholesterol, cholic acid, pregnenolone, 9-*cis*-retinoic acid (9cRA). Stereocontrolled syntheses for the following ligands were performed: 24(S),25-EC; 22(R)-hydroxy-24(S),25-EC; 24(R),25-EC; 22(S)-hydroxy-24(R),25-EC; 7-keto-, 7 β -hydroxy-, and 7 α -hydroxy-24(S),25-ECs; 24(S),25-iminocholesterol; and 22(R),24(S)-dihydroxycholesterol. The methyl ester and dimethylamide of cholenic acid were prepared from cholesterol trisnorcarboxylic acid via acid chloride by reaction with methanol and dimethylamine, respectively.

Cell Cotransfection Assays

CV-1 cell conditions and transient transfections were performed (Willy et al. 1995). Receptor expression

plasmids encoding full-length human LXR β (CMX-hLXR β) or LXR α (CMX-hLXR α) (Willy et al. 1995) were cotransfected with a luciferase reporter plasmid [TK-CYP7a-LXRE(X3)-LUC] (Peet et al. 1998) containing three tandem copies of the sequence (gcttTG-GTCActcaAGTTCAagtta) from the rat *Cyp7a* gene (Chiang and Stroup 1994). Increasing concentrations of ligand (0.1–40 mM) were added to cells in media containing 5% lipid-depleted calf bovine serum. Transfection data were normalized to a β -galactosidase internal standard.

Data are presented as mean relative light units (RLU) from triplicate assays \pm SEM. EC₅₀ values generated from duplicate assays were determined by fitting the data to a sigmoidal dose–response curve (GRAPHPAD PRISM, GraphPad Software, San Diego, Calif., USA). Efficacy values represent the fraction of maximal fold activation of each compound relative to 24(S),25-EC. Fold activation was determined by dividing the maximal activation of each compound by the activation observed in the absence of compound.

Receptor Protein Purification

Polyhistidine human RXR α (His10-hRXR α) (Petty 1995), LXR α LBD or LXR β LBD fusion proteins (His₆-hLXR α -LBD, His₆-hLXR β -LBD) were expressed in *Escherichia coli* strain BL21(DE3) (Novagen). Cultures grown in Luria-Bertani medium were induced with 0.5 mM isopropyl β -D-thiogalactoside (IPTG) for 4 h at 25°C. Pellets were suspended in lysis buffer (250 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1% Triton X-100, 10 mM imidazole, 200 mg of lysozyme per ml). The supernatant was incubated with Ni²⁺-NTA agarose (Qiagen). The resin was washed twice with 20 vols of 50 mM Hepes, pH 7.5, 250 mM NaCl supplemented first with 50 mM then 75 mM imidazole. Protein was eluted with a linear 75–500 mM imidazole gradient. Peak fractions were tested for purity by SDS-PAGE, pooled, and cleared of imidazole over a PD-10 column (Pharmacia) equilibrated with 20 mM Hepes, pH 7.5, 200 mM NaCl, 2.5 mM EDTA, 5 mM DTT. Concentrations were determined by UV spectral analysis.

Ligand-Binding Assay

Scintillant-filled beads precoated with polylysine to permit protein binding (Amersham) were diluted in scintillation proximity assay (SPA) buffer [10 mM K₂HPO₄, 10 mM KH₂PO₄, 2 mM EDTA, 50 mM NaCl, 1 mM DTT, 2 mM CHAPS, 10% (vol/vol) glycerol, pH 7.1; CHAPS is 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] to a fi-

nal concentration of 10 mg/ml. Binding assays were performed in 96-well plates (Packard) in a total volume of 100 μ l containing beads (0.2 mg per well) and His₆-hLXR α -LBD (600 ng per well), His₆-hLXR β -LBD (250 ng per well), or His₆-hRXR α (250 ng per well). The amount of protein used did not deplete ligand concentrations. [³H]-24(S),25-EC or [³H]-9cRA was diluted in SPA buffer and added to wells for a final concentration of 25 nM or 5 nM, respectively. Competition binding assays using a single concentration of unlabeled competitor contained 25 mM 24(S),25-EC or 5 μ M 9cRA. In other competition binding assays, unlabeled ligands were serially diluted in SPA buffer, then added at final concentrations ranging from 3 nM to 50 μ M. Plates were shaken at 25°C for 3 h, and then radioactivity was measured with a Packard Topcount at 1 min per well. All concentrations were assayed in triplicate and the results were averaged. Values from wells void of competitor represented 100% binding.

EVALUATION

Generation of K_i Value

Competition curves were generated by non-linear regression analysis with GRAPHPAD PRISM, and apparent equilibrium dissociation constants (K_i values) were determined by using a method described by DeBlasi et al. (1989) based on the Cheng-Prusoff equation. K_i values for compounds that served as weak competitors in the assay (competition <70% at the highest concentrations tested) are described as poor competitors. All K_i and SEM values reported are averages generated from duplicate or triplicate assays.

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M.8.3.3

Inhibition of Atherosclerosis by LXR Ligands

PURPOSE AND RATIONALE

Several authors studied the potential antiatherosclerotic effects of LXR ligands. Alberti et al. (2001) used LXR β -deficient mice to study hepatic cholesterol metabolism and resistance to dietary cholesterol.

Chawla et al. (2001) reported that the nuclear receptor LXR α mediates a transcriptional cascade via PPAR γ , which induces ABCA1 expression and cholesterol removal from macrophages. Ligand activation of PPAR γ leads to primary induction of LXR α and to coupled induction of ABCA1. Transplantation of PPAR γ null bone marrow into LDLR^{-/-} mice results in a significant increase in atherosclerosis, consistent with the hypothesis that regulation of LXR α and ABCA1 expression is protective *in vivo*.

PROCEDURE

Reagents

Oxysterols were dissolved in ethanol prior to addition to cells (<1 μ l/ml).

Cell Culture, Stable Cell Lines, and RNA Analysis

THP-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum or 10% lipoprotein-deficient fetal bovine serum (LPDS). ES cells were cultured in the presence of rLIF (Gibco) as described (Keller et al. 1993). Macrophage differentia-

tion was induced in ES cells in a two-stage process. Initially, ES cells were differentiated into embryoid bodies. Day 6 embryoid bodies were isolated, disaggregated, and replated in the presence of interleukin-3 and macrophage colony-stimulating factor-1 to induce differentiation of monocytes and macrophages, respectively. Total RNA was isolated using TRIzol reagent (Gibco), and Northern blot analyses were performed.

Transfection Assays

Murine LXR α and β promoters were amplified by PCR using the published genomic structure and sequence. Primers for PCR of LXR promoters were: LXR α : 5'-ATCCTGTCCCTTCTGTCC-3' and 5'-CC TCCAGAGTCAGCGTTC-3', and LXR β : 5'-CAGTG AGCGCATACAGGT-3' and 5'-TCTCCGACTCTGT TGCCC-3'. To determine the transcription initiation site for mABCA1, 5' RACE was performed using the 5'/3' RACE kit (Roche) with RNA from the RAW 274 cell line and mouse liver, and the gene-specific 3' primer (5'-CTGAGGCCAACAAGCCAT-3'). The extended RACE products were used to screen a mouse genomic library (Stratagene). Sequence analysis of the isolated clones by the Blast program revealed that the identified initiation site and 5' regulatory region of ABCA1 were identical to the sequences deposited in GenBank (accession number AJ017356). The 2.5-kb genomic fragment of the mABCA1 promoter was cloned into a pGL3-basic vector. CV-1 cells were transfected by lipofection and assayed for reporter activity as described by Forman et al. (1995). Transfections were performed in triplicate and normalized to an internal CMX- β gal control. pCMX expression vectors for the PPARs and LXRs have been described (Kliwer et al. 1994; Willy et al. 1995).

Gel Mobility Shift Assays

Gel mobility shift assays were performed using *in vitro* translated receptors and 32P end-labeled oligonucleotides in a buffer containing 20 mM HEPES (pH 7.4), 100 mM KCl, 1 mM β -mercaptoethanol, 10% glycerol, 100 mg/ml polydI-dC, and 5 mg/ml BSA. For competition studies, an excess of unlabeled oligonucleotide was added.

Cholesterol Efflux

Cholesterol efflux assays were performed as described by Venkateswaran et al. (2000), with minor modifications. Cells were plated at 50% confluence. On day 2, cells were washed and incubated for 24 h in

RPMI 1640 supplemented with 10% LPDS. Cells were labelled with [³H]cholesterol (1.0 mCi/ml), either in the presence of the ACAT inhibitor (58-035; 2 μ g/ml) or with acLDL (50 μ g/ml) in the absence of the ACAT inhibitor. Ligands for PPAR β (BRL; 5 μ M), RXR (LG268; 50 nM), or LXR (22[R]-OHC; 2.0 μ g/ml) were added to the cells. To equilibrate cholesterol pools, cells were washed twice with PBS and incubated for 8 h in RPMI containing 0.2% BSA plus the ligands, but lacking radiolabeled cholesterol or acLDL. Cells were again washed with PBS and incubated in RPMI containing 0.2% BSA in the absence or presence of HDL (50 μ g/ml) or apoAI (15 μ g/ml), for 4 h. An aliquot of the medium was removed and centrifuged at 14,000 g for 2 min, and the radioactivity was determined by liquid scintillation counting. Total cell-associated radioactivity was determined by dissolving the cells in isopropanol.

The data are presented as percent HDL-specific efflux or apoAI-specific efflux, which is efflux in the presence of acceptor minus efflux in the absence of acceptor. Each assay was performed in triplicate.

Bone Marrow Transplantation

LDL-R^{-/-} mice backcrossed onto the C57BL/6 background were initially obtained from Jackson Laboratories. Mice were fed a chow diet ad libitum until they were enrolled into the study. PPAR γ 2/2ES cells were injected into C57/Bl6 blastocysts to generate PPAR γ ^{-/-} chimeric mice. The majority of the chimeric mice obtained were high-percentage chimeras. Peripheral blood, spleen, and bone marrow of PPAR γ ^{-/-} chimeras were extensively analyzed by facs analysis with antibodies directed against Ly 9.1, CD11b/CD18, F4/80, CD3, B220, and Ly 6.1 to quantify the extent of chimerism. Chimeras, which were greater than 90% null in their contribution to the monocytic/macrophage lineage, were used for bone marrow transplantation, along with wild-type control mice. In all, 24 6-week-old male LDL-R^{-/-} mice were γ -irradiated with 1000 rads to eliminate the majority of their bone marrow cells. Each of the γ -irradiated mice were reconstituted via a tail vein injection with 2,000,000 bone marrow cells isolated from either PPAR γ +/+ mice (designated PPAR γ +/+ BMT) or PPAR γ 2/2 mice (designated PPAR γ 2/2 BMT). The mice were placed on a chow diet for 4 weeks while their marrow repopulated with the donor cells. For induction of atherosclerosis, the transplanted mice were placed on an atherogenic diet containing 15.8% (wt/wt) fat and 1.25% cholesterol (no cholate; diet 94059, Harlan Teklad) for 8 weeks. At weeks 0 (before BMT), 4, and 12, blood was drawn via

the retro-orbital plexus following an 8-h fast, and total plasma cholesterol was measured via an enzymatic assay.

Lesion Analysis and Immunohistochemistry

Atherosclerotic lesions were quantified in the aortic valve of each mouse as described by Boisvert et al. (1998). The OCT-embedded, frozen aortic valves were sectioned serially at 10 μm thickness for a total of 300 μm beginning at the base of the aortic valve, where all three leaflets are first visible. Every fourth section for a total of five sections from each animal was stained with oil red O to identify the lipid-rich lesions. The stained areas were quantified using a computer-assisted video imaging system, and the mean area of the five sections from each animal was used for comparison analysis. The mouse aortic valve lesions were analyzed immunohistochemically with the following antibodies: anti-MOMA-2 (Serotec) for detection of intimal macrophages; anti- β -galactosidase (Cappel) for detection of PPAR γ ^{-/-} cells; and anti- α -actin (Dako) for detection of smooth muscle cells in the lesion. The frozen tissue sections were blocked with 5% normal sera and incubated for 2 h at room temperature with the primary antibody (1–10 $\mu\text{g}/\text{ml}$). The sections were blocked for endogenous peroxidase activity with Peroxo-Block (Zymed, South San Francisco, Calif., USA), followed by incubation with the appropriate secondary antibody (5 $\mu\text{g}/\text{ml}$) for 1 h. The washed sections were finally incubated for 30 min with Vectastain ABC Elite solution (Vector Laboratories, Burlingame, Calif., USA), developed with 9-amino-3-ethylene-carbazole (AEC; Vector Laboratories), and counterstained with hematoxylin.

EVALUATION

Statistical analysis was performed using the Mann-Whitney *U* test, and data are expressed as mean \pm SD.

MODIFICATIONS OF THE METHOD

Dressel et al. (2003) used mouse myogenic C2C12 cells to study the influence of a peroxisome proliferator-activated receptor β/δ agonist on the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells.

Tanaka et al. (2003) used skeletal muscle cells of mice to study the influence of peroxisome proliferator-activated receptor δ on fatty acid β -oxidation in skeletal muscle and the metabolic syndrome.

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Safety Pharmacology of Anti-Atherosclerotic Drugs

See Herling (2006).

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Chapter N

Endocrinology¹

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N.1 Preface

In the development of methods for endocrine investigation, many changes have occurred since the early period of animal investigation, in which extensive surgical procedures were used to remove endocrine organs in order to study their function by deficiency symptoms, and their active principles were replaced by organ extracts and hormone preparations of increasing purity and specificity of action. Much of the classical endocrinology has now been replaced by investigations of ever increasing sophistication and specificity. Where possible, surgical procedures are discarded and avoided. Studies are performed on intact animals with a minimum of stress and interference (noninvasive procedures). One example of early noninvasive investigation is the study of adrenal gland hormone secretion, by investigation of the urinary excretion of adrenal steroids and changes induced by specific procedures.

Where surgical procedures cannot be avoided, techniques for anesthetizing animals have changed very markedly (new anesthetics have replaced ether anesthesia), animal husbandry has undergone major changes, and postoperative animal care has been standardized and optimized. Specific strains of animals with endocrine disease conditions have been developed by selection from naturally occurring genetic disorders, and increasingly detailed investigations are now available through the study of genetics and patterns of gene expression following the administration of test compounds and candidate drugs.

There is however a price to be paid for any method of increasing sophistication. The interpretation of the relevance of changes to the clinical situation (predictive relevance) becomes more difficult. As an example, in the chain of events from receptor binding of a drug, to the intracellular changes summarized as re-

ceptor signaling, and finally to the observed effects on patterns of gene expression, interpretation is much more tenuous and the pathway to understanding clinical relevance is often more difficult than it used to be. Endocrinologists will have to be careful and conservative when interpreting their findings, and often a set of coordinated investigations is needed before the complex early findings of an investigation can be unraveled. With these words of introduction, the following descriptions of methods are presented to encompass both historical and classical approaches, as well as the highly effective and specific investigation of detail now at hand.

N.1.1 Endocrine Survey

PURPOSE AND RATIONALE

The effects of steroid and peptide hormones as well as the influence of drugs on the endocrine system are detected by repeated administration of the test substances. The procedure is analogous to toxicology studies with repeated administration, however the dose range is limited to the intention of understanding physiological changes at concentrations of endogenous compounds found in the body, and concentrations of drugs which are intended for future therapeutic use (methods with intention to predict clinical effects and explain physiological regulations) (Sandow 1979). Animals are treated for at least several days to achieve equilibration of the physiological response to administration of compounds. After administration for a period of 1–4 weeks, the endocrine glands and the organs that are dependent on hormonal influences are weighed. Furthermore, the hormonal content of the endocrine glands and the blood levels of hormones are determined.

During treatment and at the time of animal sacrifice, blood samples or tissue samples may be obtained where needed for investigation and interpretation, and frequently the same treatment protocol may need to be repeated when taking samples during treatment, and when investigating the time sequence of induced changes, always with a minimum of interference and based on noninvasive procedures whenever possible.

PROCEDURE

The endocrine survey is performed using young or adult rats depending on the purpose of the investigation. Selection of this animal species is based on extensive knowledge of physiological data for organ development and hormone secretion. It is also based on the

technology for obtaining blood and urine samples with a minimum of stress to the animals. Separate groups of five to ten male and female Sprague-Dawley rats weighing 55–65 g are used (animals during development). For some compounds, groups of 200 or 300 g body weight may be required. They are treated daily over a period of 6–12 days with the test compound by the intended route (orally or by subcutaneous injections). For toxicological studies, a treatment period of 4 weeks is preferable. A similar protocol is applied for chronic toxicity studies in rats and dogs. On the day after the last application, the animals are sacrificed, weighed, and the parameters determined:

| Parameter | Optional |
|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Pituitary-adrenal axis | |
| Pituitary weight | Corticotropin-releasing hormone in hypothalamus |
| Adrenal weight | Pituitary content of adrenocorticotropin and vasopressin |
| Thymus weight | Adrenal content of corticosterone and aldosterone; corticosterone and aldosterone blood level; electrolyte (Na^+ , K^+ , Ca^{2+} , Cl^-) concentrations in serum; urinary excretion of corticosterone and aldosterone |
| Pituitary-gonadal axis | |
| <i>Male reproduction</i> | |
| Pituitary weight | LH-RH in hypothalamus |
| Weight of testes | Pituitary content of FSH, LH and prolactin |
| Weight of seminal vesicles | Blood levels of FSH, LH and prolactin |
| Weight of ventral prostate | Testosterone content in testes and serum |
| Weight of musculus levator ani | Growth hormone in pituitary and serum |
| <i>Female reproduction</i> | |
| Pituitary weight | LH-RH in hypothalamus |
| Weight of ovaries | Pituitary content of FSH, LH and prolactin |
| Weight of uterus | Blood levels of FSH, LH and prolactin; content of estradiol and progesterone in ovaries and serum |
| Pituitary-thyroid axis | |
| Pituitary weight | TRH in hypothalamus |
| Thyroid weight | Pituitary content of thyrotropin; serum content of TSH, T3 and T4 |

FSH Follicle-stimulating hormone, *LH* luteinizing hormone, *LH-RH* LH-releasing hormone, *TRH* thyrotropin-releasing hormone

After dissection, organ weights are recorded, and serum or plasma and the endocrine glands are stored frozen at -20°C for further determination of hormonal content. This method may include histology, in situ hybridization, etc. as required. In many instances, an investigation of patterns of gene expression can now be part of these studies, in particular when previous experience is available for the changes considered to be of relevance.

EVALUATION

The mean values of each parameter for the treated groups are compared with the values for the control group. The control group is generally treated with the solvent or vehicle required for administration of the test compound. In many cases a group of untreated animals, undergoing the same sampling procedures, is investigated in order to make certain of the physiological background for comparison.

MODIFICATIONS OF THE METHOD

Many modifications are possible; for example, extension of the treatment period up to 4 weeks in order to detect toxicological effects after long-term administration, inclusion of supplementary parameters, satellite groups. Further specific tests are necessary to clarify the mode of action.

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N.2

Adrenal Steroid Hormones

N.2.0.1

Adrenalectomy in Rats

PURPOSE AND RATIONALE

The classical way to evaluate hormone function is to surgically ablate the hormone-producing endocrine gland and substitute with exogenously administered substances (extracts or synthetic hormones) (Biedl 1916). Many studies on the physiological role of adrenocortical hormones and the pharmacological effects of corticosteroids were performed using adrenalectomized rats.

PROCEDURE

Sprague-Dawley or Wistar rats of either sex weighing 120–150 g are used. The dorsal fur is shaved, the rat

is anesthetized and placed on a support in order to elevate the viscera. A transverse incision about 5 mm long is made in the mid-line at the costovertebral angle. To remove the left adrenal gland, the skin is retracted to the ventral side and the lumbar muscles incised just superior and anterior to the splenic shadow. The adrenal gland now appears directly beneath the incision and no exteriorization of the kidney is necessary. The periadrenal tissue is grasped between the kidney and the adrenal by small curved forceps and the intact gland together with the periadrenal fat and the mesenteric attachments is removed in toto. The bleeding is negligible in young animals so that no vessels need be tied off.

After ablation of the left adrenal, the animal is turned around and the right gland is removed through the original skin incision. A small incision through the lumbar muscles is made just above and anterior to the prominent lumbocostal arch which is seen near the costal margin. The curved forceps are inserted over the kidney and by elevating the liver, which covers the adrenal on this side, the gland is brought into view and grasped by the forceps, removing again the intact gland with the periadrenal fat and the mesenteric attachments. The incisions made in the lumbar muscles need not exceed 3 mm in length and may be made by spreading the blades of a pair of scissors; hemostasis or closure by sutures is not necessary. The incision is closed by a skin clip. The entire procedure is done in a time sufficiently short to avoid long-acting anesthetics. The animals appear normal in every aspect within a few minutes following the operation.

It is essential to provide good postoperative care for animals after adrenalectomy, and to correct the electrolyte imbalance due to deficiency of mineralocorticoid secretion (1% sodium chloride solution in water).

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N.2.1

Glucocorticoid Activity

N.2.1.1

In Vitro Methods for Glucocorticoid Hormones

N.2.1.1.1

Corticoid Receptor Binding

PURPOSE AND RATIONALE

Steroid hormone receptors are intracellular metallo-proteins which bind steroids with high affinity and high specificity (nuclear receptors). The lipophilic steroids enter the cell by diffusion. The binding of the hormone to its specific receptors results in a series of conformational changes, leading to an increase in affinity for specific DNA regulatory elements (steroid responsive elements). After binding, transcription of target genes is enhanced and mRNAs are produced. These mRNAs are then translocated to the cytoplasm and translated into proteins regulating cellular metabolism and initiating the cellular responses. The structure and functions of the steroid receptor superfamily have been reviewed by Carson-Jurica et al. (1990), Lazar (1991), Barnes and Adcock (1993), Distelhorst (1993), Power et al. (1993), Brinkmann (1994), Ojasoo et al. (1994, 1995), Wittliff and Raffelsberger (1995), Beato et al. (1996), and Jensen (1996). The relative binding affinities for the glucocorticoid receptor of rat liver or thymus cytosol can be measured by competitive displacement of [³H]dexamethasone (Raynaud et al. 1979; Schlechte et al. 1982; Wojnar et al. 1986; Ueno et al. 1991).

PROCEDURE

A steroid receptor preparation may be obtained from rats. Male Wistar rats weighing 140–150 g are adrenalectomized under anesthesia. Two days later, the liver is excised and homogenized in a tenfold buffer containing 50 mM Tris-HCl, 1 mM EDTA, 2 mM dithiothreitol, 10 mM Na₂MoO₄, and 10% glycerol (pH 7.4). The homogenate is centrifuged for 1 h at 105,000 g at 4°C and collected as the supernatant fraction. The cytosol is mixed with 5 nM [³H]dexamethasone in the presence or absence of appropriate concentrations of competitors and incubated for 2 h at 4°C. The reaction is terminated by the addition of hydroxyapatite in order to separate the receptor–steroid complex from the free [³H]dexamethasone.

The radioactivity bound to the receptors is determined by liquid scintillation spectrometry.

EVALUATION

IC_{50} values for the receptor preparation are calculated by probit analysis.

MODIFICATIONS OF THE METHOD

Numerous modifications of the glucocorticoid receptor assay have been reported, and cloning of receptor subtypes is ongoing (Zeelen 1992; White et al. 1994). Cytosols have been prepared from rat liver, from cultured rat hepatoma cells (Rousseau and Schmit 1977), and from normal human lymphocytes (Steiner and Wittliff 1985); thymocytes, from rat thymus gland (Lefebvre et al. 1988), human leukemic lymphoid cell line, CEM C7 (Srivastava and Thompson 1990), rat lung (Druzgala et al. 1991; Hochhaus et al. 1991), human lung (Rohdewald et al. 1985), rat hippocampus (Jacobson et al. 1993) and other sources.

Differentiation of type I (mineralocorticoid) and type II (glucocorticoid) receptors has been attempted (Spencer et al. 1990; Jacobson et al. 1993).

A new affinity label for glucocorticoid receptors was described by Lopez and Simons (1991).

The solution structure of the glucocorticoid receptor DNA-binding domain has been determined (Härd et al. 1990).

Berger et al. (1992) used transient co-transfection of receptor cDNA and suitable reporter genes to study human glucocorticoid receptor function in a CV-1 mammalian cell line. A variety of natural and synthetic steroids were analyzed for their ability to activate gene expression through the human glucocorticoid receptor and to bind to extracts of cells expressing human glucocorticoid receptor cDNA. A good correlation between both *in vitro* parameters and *in vivo* anti-inflammatory activity was reported for most of the steroids tested.

Guo et al. (1995) tested the binding of various steroids to the synaptic plasma membrane, which may be a novel type of glucocorticoid receptor on neuronal membranes that is significantly different from cytosolic glucocorticoid receptors.

A general survey of steroid hormones, receptors and antagonists was given by Jensen (1996).

Teutsch et al. (1995) discussed the structure-activity correlation of hormone antagonists ("anti-hormones").

Yoshikawa et al. (2002) showed that the pyrazolosteroid cortivazol is a specific ligand for the glucocorticoid receptor.

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N.2.1.1.2

Transactivation and Transrepression Assays for Glucocorticoids

N.2.1.1.2.1

General Considerations

PURPOSE AND RATIONALE

The effects of glucocorticoids are mediated by both transactivation and transrepression mechanisms. Classical glucocorticoids, such as prednisolone or dexamethasone, induce both mechanisms. The desired anti-inflammatory effects are mainly mediated by repression of gene expression, whereas side-effects are predominantly mediated via transactivation (Pfahl 1993; Sch acke et al. 2002; Buttgerit et al. 2005).

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N.2.1.1.2.2

Transactivation Assay for Glucocorticoids

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows determination of the agonistic and also the antagonistic potency of a given compound, by induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

PROCEDURE

Vector Construct

The expression plasmid pHGO containing the full-length coding sequence of the human glucocorticoid receptor driven from the SV 40 early promoter is prepared according to Hollenberg et al. (1985). The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

CV-1 cells and COS-1 cells for transfection are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 4 mmol/l L-glutamine, penicillin and streptomycin. Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989), and Felgner et al. (1987). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80% confluent after 24 h.

Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg pHGO (hGR expression plasmid) and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10% fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3% charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1% ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37°C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982). After the cells are spun for 15 min in an Eppendorf microfuge at 4°C, the supernatants are removed and assayed for enzyme activity. The assay mixture contains (in a final volume of 180 µl) 100 µl of 0.25 M Tris-hydrochloride (pH 7.5), 20 µl of cell extract, 1 µCi of [¹⁴C]chloramphenicol (50 µCi/mmol; New England Nuclear), and 20 µl of 4 mM acetyl coenzyme A. Controls contain CAT (0.01 U; P.L. Biochemicals) instead of cell extract. All of the reagents except coenzyme A are pre-incubated together for 5 min at 37°C. After equilibration is reached at this temperature, the reaction is started by adding coenzyme A. The reaction is stopped with 2 ml cold ethyl acetate, which is also used to extract the chloramphenicol. The organic layer is dried and taken up in 30 µl of ethyl acetate, spotted on silica gel thin-layer plates, and run with chloroform:methanol (95:5), ascending. After autoradiography of the separated acetyl chloramphenicol forms, the spots are cut out and counted. Data are expressed as the amount of chloramphenicol acetylated by 20 µl of extract.

EVALUATION

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to determine the potency of the test hormone. Dexamethasone (10^{-10} – 10^{-6} mol/l) serves as standard.

For antiglucocorticoid activity, CAT activity in the presence of 10^{-8} mol/l dexamethasone is set as 100% and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the steroid hormone antagonist RU 486 as the standard inhibitor.

CRITICAL ASSESSMENT OF THE METHOD

Hollon and Yoshimura (1989) examined the causes of high variability in data from enzymatic transient gene expression assays. Their results strongly suggested that variation in transfection efficacy is the major cause of data variation and can seriously compromise valid interpretation of data.

MODIFICATIONS OF THE METHOD

White et al. (1994) described a simple and sensitive high-throughput assay for steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high levels of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells using a colorimetric chloramphenicol acetyltransferase assay.

Dias et al. (1998) recommended the use of CRE recombinase in mammalian cells for high throughput screening of chemical libraries to identify new receptor ligands. A translational fusion of CRE recombinase and the ligand binding domain of the human glucocorticoid receptor was transfected into mammalian cells with the LOX P/luciferase reporter gene (DeWet 1907). A stable transfected clone was isolated and

used to characterize the kinetics, ligand specificity, and dose–response to various receptor ligands.

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N.2.1.1.2.3

Transrepression Assay for Glucocorticoids

PURPOSE AND RATIONALE

The immunosuppressive and anti-inflammatory actions of glucocorticoid hormones are mediated by transrepression of activating protein-1 (AP-1) and nuclear factor kappa B (NF κ B) transcription factors. Since the anti-inflammatory effects of glucocorticoids are thought to be related to repression mechanisms and the

side-effects mainly to activation mechanisms, a therapeutic benefit of dissociated glucocorticoids is expected (Vayssière et al. 1997; Vanden Berghé et al. 1999; Belvisis et al. 2001; Lin et al. 2002; Tanigawa et al. 2002; Schottelius et al. 2002, Coghlan et al. 2003; Lietal 2003; Ali et al. 2004; Hochhaus 2004; Schäke et al. 2004; Smith et al. 2005; Thompson et al. 2005). Schäke et al. (2004) investigated the dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist, leading to separation of therapeutic effects from side-effects.

PROCEDURE

Receptor-Binding Assays

Cytosol preparations of Sf9 cells, infected with recombinant baculovirus coding for the human GR, MR, or PR, were used for the binding assays. After centrifugation (15 min, 600 g) Sf9 pellets were resuspended in 1/20 volume of 20 mM Tris-HCl, pH 7.5; 0.5 mM EDTA, 2 mM DTT, 20% glycerol, 400 mM KCl, 20 mM sodium molybdate, 0.3 μ M aprotinin, 1 μ M pepstatin, 10 μ M leupeptin and shock frozen in liquid nitrogen. After three freeze–thaw cycles, the homogenate was centrifuged for 1 h at 100,000 g. The protein concentration in the resulting supernatant was between 10 and 15 mg/ml. Aliquots were stored at -40°C .

For the binding assays for GR, MR, and PR, [^3H]dexamethasone (≈ 20 nM), [^3H]aldosterone or [^3H]progesterone, Sf9 cytosol (100–500 μ g protein), test compound, and binding buffer (10 mM Tris-HCl, pH 7.4; 1.5 mM EDTA, 10% glycerol) were mixed in a total volume of 50 μ l and incubated for 1 h at room temperature. Specific binding was defined as the difference between binding of [^3H]dexamethasone, [^3H]aldosterone, and [^3H]progesterone in the absence and presence of 10 μ M unlabeled dexamethasone, aldosterone, and progesterone.

After incubation, 50 μ l of cold charcoal suspension was added for 5 min, and the mixtures were transferred to microtiter filtration plates. The mixtures were filtered into Picoplates (Canberra Packard, Dreieich, Germany) and mixed with 200 μ l of Microszint-40 (Canberra Packard). The bound radioactivity was determined with a Packard Top Count plate reader. The concentration of test compound giving 50% inhibition of specific binding (IC $_{50}$) was determined from Hill analysis of the binding curves.

Induction of Tyrosine Aminotransferase (TAT)

Induction of TAT by test compounds was determined *in vitro* in the rat liver hepatoma cell line H4-II-

EC3. Cells were grown in MEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), and 1% NEAA (nonessential amino acids) (Life Technologies). For compound testing, cells were seeded in 96-well plates (2×10^4 cells per well) and incubated with test compounds or dexamethasone for 20 h. Cells were then lysed and TAT activity assayed as described below for hepatic TAT induction *in vivo*.

Inhibition of IL-8 Production

IL-8 synthesis was induced by stimulation of the human promyelocytic cell line THP-1 with lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8; Sigma). Cells (2.5×10^4 cells per well) were treated with 10 $\mu\text{g}/\text{ml}$ LPS in the absence or presence of test compounds or dexamethasone for 18 h. IL-8 concentration was determined in the supernatant by an IL-8-specific ELISA (Beckman Coulter).

Inhibition of Monokine Secretion

Monocytic secretion of tumor necrosis factor- α (TNF- α) and IL-12 p70 was determined after stimulation of peripheral blood mononuclear cells from healthy donors with 1 $\mu\text{g}/\text{ml}$ LPS (*E. coli* serotype 0127:B8; Sigma) and 10 ng/ml interferon- γ 1b (IFN- γ 1b) (Imukin, Boehringer Ingelheim). After 24 h of stimulation at 37°C, 5% CO₂, in the absence or presence of different concentrations of the compounds, the concentrations of IL-12 p70 and TNF- α in culture supernatants were determined by using commercial ELISA kits from R and D Systems (IL-12 HS Immunoassays, Nivelles, Belgium) and Bio-Source International (TNF- α EASIA, Wiesbaden, Germany). The calculated IC₅₀ value represents the concentration of compound giving 50% inhibition of maximal TNF- α and IL-12 p70 production.

MODIFICATIONS OF THE METHOD

Vayssière et al. (1997) described synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression as being weak transactivators of tyrosine aminotransferase (TAT) in cultured liver cells but potent inhibitors of LPS-induced IL-1 β secretion in the human monocytic cell line THP1.

González et al. (2000) found that glucocorticoids antagonize AP-1 by inhibiting the activation/phosphorylation of JNK without affecting its subcellular distribution.

Kagoshima et al. (2001) reported that glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation.

Reichardt et al. (2001) studied the repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor using mice with a mutation in the glucocorticoid receptor, which cannot activate glucocorticoid receptor response element promoters.

De Haij et al. (2003) reported dissociation of transrepression and transactivation in the steroid responsiveness of renal epithelial cells.

Schaaf and Cidlowski (2003) investigated the importance of ligand affinity for molecular determinants of glucocorticoid receptor mobility in living cells.

Stevens et al. (2003) investigated the role of tyrosine 735 in the dissociation of steroid receptor co-activators and nuclear receptor recruitment to the human glucocorticoid receptor.

Carballo-Jane et al. (2004) recommended skeletal muscle cells as a dual system to measure glucocorticoid-dependent transactivation and transrepression of gene regulation.

Studying transactivation and transrepression activity, Koubovec et al. (2005) found that synthetic progestins used in hormone replacement therapy have different glucocorticoid agonist properties.

Eberhardt et al. (2005) reported that dissociated glucocorticoids equipotently inhibit cytokine- and cAMP-induced matrix-degrading proteases in rat mesangial cells.

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N.2.1.1.3

Induction of Tyrosine Aminotransferase (TAT) in Hepatoma Cells

PURPOSE AND RATIONALE

The synthesis of tyrosine aminotransferase in hepatoma tissue culture (HTC) cells (Thompson et al. 1966) can be induced by glucocorticoids (Giesent and Beck 1982; Raynaud et al. 1980; Rousseau et al. 1977). This effect can be abolished by glucocorticoid antagonists.

PROCEDURE

Hepatoma tissue culture (HTC) cells are grown in suspension to a density of about 8×10^5 cells/ml. The cells are washed 3 times at 0°C in a total volume of buffered saline equivalent to half of the culture medium. The suspension is centrifuged and cell pellets resuspended in serum-free medium containing 0.1% BSA and 0.1% NaHCO₃. Ethanol solutions of standard (dexamethasone) and test steroids in various concentrations are added to 10-ml aliquots of cell suspension, with the final ethanol concentration not exceeding 0.5%. After 16 h of incubation in tightly capped flasks on a rotary shaker (100 rev/min) at 37°C, the cells are harvested and tyrosine aminotransferase determined. The cells are washed twice and then disrupted with an ultrasonicator. The enzyme is assayed at 37°C by conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde (Diamondstone 1966). One unit of activity represents the conversion of 1 μ mol of *p*-hydroxyphenylpyruvate per minute.

EVALUATION

Enzyme-specific activity is expressed in milliunits of tyrosine aminotransferase/mg of cell protein. Dose-response curves are established for the standard

and the test preparation allowing the calculation of potency ratios with confidence limits.

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N.2.1.1.4

Effect on T-Lymphocytes

PURPOSE AND RATIONALE

Corticosteroids as immunosuppressive agents have suppressive effects on T-lymphocyte function. Snijdwint et al. (1995) determined *in vitro* dose-dependent inhibition of Th1- and Th2-type cytokine production by various corticosteroids.

PROCEDURE

Culture Media

T-lymphocyte clones are maintained and expanded in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% pooled c-inactivated human serum and gentamicin (80 μ g/ml). Experiments are performed in IMDM supplemented with 10% fetal calf serum (FCS), human transferrin (35 μ g/ml), 2-mercaptoethanol (2-ME, 3.5 μ g/l) and human insulin (1.75 IE/ml).

Peripheral Blood Lymphocytes and T-Lymphocyte Clones

Peripheral blood mononuclear cells are prepared from heparinized venous blood of healthy volunteers by flotation on Lymphoprep (Nycomed, 1.077 g/ml) and the peripheral blood lymphocytes are separated by centrifugation on a discontinuous Percoll density gradient. Peripheral blood lymphocytes are collected from the interface between densities 1.977 g/ml and 1.061 g/ml, washed three times in HBSS plus 2% FCS

and suspended in IMDM plus 10% FCS. Isolated peripheral blood lymphocytes (0.5×10^6 cells/ml) are stimulated with CD2 mAb plus CD28 mAb (1:1000 final dilution of ascites) in the absence or presence of corticosteroids (Van der Pouw-Kraan et al. 1992). Supernatants are collected after 48 h for IFN- γ and IL-2 measurement, and after 96 h for IL-4 measurement.

T-lymphocyte clones specific to housedust mite (*Dermatophagoides pteronyssinus*) are generated from the peripheral blood and skin of atopic individuals (Van der Heijden et al. 1991). These clones are Th0 typed, capable of producing both IFN- γ and IL-4 (Kapsenberg et al. 1988). T-lymphocyte clone cells (0.5×10^6 cells/ml) are stimulated with CD2 and CD28 mAb (1:1000) plus 1 ng/ml phorbol 12-myristate 13-acetate (PMA) to induce optimal levels of cytokine production. Corticosteroids are added at the start of the stimulation and then 24 h after stimulation, supernatants are collected and stored at $\approx 20^\circ\text{C}$ until cytokine production is analyzed.

Proliferation Assay and Cytokine Measurements

Proliferation by peripheral blood lymphocytes is measured 48 or 96 h after stimulation in 96-well flat-bottom culture plates using 10^4 cells/well, the last 16 h in the presence of 13 kBq (0.33 μ C)/well of [^3H]TdR (Radiochemical Centre, Amersham, UK). The proliferation of T-lymphocyte clones (10^4 cells/well) is similarly measured after stimulation for 40 h, again the last 16 h in the presence of [^3H]TdR. Incorporation of [^3H]TdR is determined by liquid scintillation spectroscopy.

Measurement of IFN- γ and IL-4 in peripheral blood lymphocytes and T-lymphocyte clone supernatants is performed with specific solid-phase sandwich ELISA systems (Van der Pouw-Kraan et al. 1992). For quantification of IL-2 production, a commercial ELISA kit is used. IL-2R expression by T cells of peripheral blood lymphocytes is determined 48 h after stimulation by labeling with mouse anti-human CD25 mAb.

EVALUATION

Dose-response curves of inhibition of IFN- γ , IL-4 and IL-2 production as a percentage of initial values in peripheral blood lymphocytes and in different T-lymphocyte clones are established for various corticosteroids in the range of 10^{-10} to 10^{-6} M.

All measurements are performed in duplicate or triplicate. Data are expressed as mean \pm SEM and differences are assessed using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Mollison et al. (1999) compared a macrolactam inhibitor of T helper type 1 and T helper type 2 cytokine biosynthesis in various animal models with several steroid preparations used for topical treatment of inflammatory skin diseases.

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N.2.1.1.5

Inhibition of Cartilage Degradation

PURPOSE AND RATIONALE

The efficacy of glucocorticosteroids after intra-articular injection has been clearly demonstrated in several animal models of osteoarthritis (Van den Berg et al. 1992; Pelletier et al. 1995). Augustine and Oleksyszyn (1997) used *in vitro* experiments to study the inhibition of degradation in bovine cartilage explants stimulated with concomitant plasminogen and interleukin-1 α by various glucocorticosteroids.

PROCEDURE

Preparation of Bovine Articular Cartilage Explants

Bovine (calf) radiocarpal joints are acquired from a local abattoir immediately after sacrifice and transported on ice. The specimens are washed thoroughly and placed on ice containing 25% Povidine (10% Povidone-iodine topical solution). The specimens are then dissected in a sterile hood using good sterile technique. Media (DMEM containing 4.5 g/l D-glucose and L-glutamine, without sodium pyruvate) is supplemented with HEPES buffer (3.57 g/l) and sodium

bicarbonate (3.7 g/l), and the pH adjusted to 7.4. The medium is further supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml) and 50 μ g/ml L-ascorbic acid. The articulating cartilage surfaces are exposed, and the synovial fluid is wiped away with sterile gauze. A sterile cork-borer with a diameter of 3.5 mm is used to remove uniform cores of cartilage. The cores are placed in a sterile flask, washed 4 times with sterile medium, and then placed in an incubator (37°C, 5% CO₂/95% air, adequate humidity) and allowed to equilibrate for 1 h. The cartilage disks are then labeled en mass with [³⁵S]sulfate at 10 μ Ci/ml for approximately 72–96 h, with hand-stirring every few hours. After labeling, explants are equilibrated with fresh medium (minimum of two washes before use in experiments).

Inhibition of IL-1-Induced Cartilage Degradation in Bovine Articular Cartilage in the Presence of Human Plasminogen

Individual explants are transferred to 96-well plates containing 250 μ l of fresh medium per well, with or without plasminogen and IL-1 α , and with or without glucocorticosteroids. A negative control consists of medium alone, while two positive controls are IL-1 α alone, and plasminogen with IL-1 α . All other groups contain the glucocorticosteroids along with recombinant human IL-1 α (0.4 ng/ml) alone or concomitant human plasminogen (0.4 μ M) plus IL-1 α . Glucocorticosteroids are added in concentrations between 10 and 10,000 pM. Control and experimental explants are incubated for approximately 96 h (4 days) prior to counting a 50- μ l sample of supernatant from each well. A 50- μ l sample of a papain-digest of each explant is also counted.

EVALUATION

The data are expressed as percentage glycosaminoglycan release over the 4 days. The values for all groups with glucocorticosteroids present are then compared to the values for concomitant plasminogen and IL-1 α without glucocorticosteroids, and percentage inhibitions are calculated. Dose–response curves are established for each glucocorticosteroid.

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N.2.1.2

In Vivo Methods for Glucocorticoid Hormones

N.2.1.2.1

General Considerations

Muscle work tests (early fatigue) were used in early stages of adrenal gland research (Bomskov 1937; Ingle 1944; Dorfman 1962), and their description is of historical interest. The method is based upon the fact that muscular responsiveness is lost within a few hours following the removal of the adrenal glands and can be maintained by the administration of corticosteroids. Since the 11-oxygenated steroids are quite active and compounds such as desoxycorticosterone are practically without activity, it appears that the main effect monitored is on carbohydrate metabolism.

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N.2.1.2.2

Adrenal and Thymus Involution

PURPOSE AND RATIONALE

Repeated administration of corticosteroids causes central and peripheral effects on pituitary hormone content and secretion, and on adrenal weight and histology. In the immature rat, thymus involution is observed in a dose-related manner.

PROCEDURE

Groups of six to ten immature male Sprague–Dawley rats weighing 60–70 g are injected subcutaneously daily for 6 days with 0.2 ml of a homogenized suspension of different doses of the test compound in 0.5% aqueous carboxymethylcellulose solution. The standard, hydrocortisone acetate, is given in daily doses of 0.05 and 0.2 mg per animal. Controls receive

the vehicle. On the 7th day, the animals are sacrificed and the adrenal and thymus weights determined.

EVALUATION

The involution of the thymus gland is a measure of the catabolic activity of the compound (metabolic effect). Involution of the adrenals is a measure of the ability of the compound to inhibit the secretion of ACTH (negative steroid feedback). Dose–response curves are established for both parameters and compared with hydrocortisone and the potency ratios are calculated.

MODIFICATIONS OF THE METHOD

The numeric test result is improved when the product of thymus and adrenal weights is calculated. Dose–response curves of the product of thymus and adrenal weights are steeper than those of each individual parameter (Laschet and Hohlweg 1960).

Inhibition of uridine incorporation into the ribonucleic acid of thymocytes has been used as a parameter for agonistic and antagonistic glucocorticoid activity (Gagnault et al. 1977).

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N.2.1.2.3

Eosinopenia in Adrenalectomized Mice

PURPOSE AND RATIONALE

Glucocorticoids decrease the eosinophilic cell blood count in laboratory animals as well as in humans. This effect was used to quantitate the potency of corticosteroids in adrenalectomized mice (Silber and Arcese 1964; Speirs and Meyer 1951; Tolksdorf 1959).

PROCEDURE

Male mice weighing 20–25 g (e. g., of Jax C57 Brown, subline cd strain) are adrenalectomized and maintained at 28°C with 1% sodium chloride solution in water.

Then, 15-mg pellets of desoxycorticosterone acetate are implanted at the time of operation. Steroids are dissolved in benzyl alcohol and mixed with sesame oil (1:10). Three days after operation, the mice receive 5 µg epinephrine by subcutaneous injection and 4 h later 1–6 µg of hydrocortisone (or the test substance) in 0.03 ml oil. Three doses of the unknown substance and standard are tested, using six animals per dose. Blood samples are taken from the tail before and 3 h after steroid injection. Mice with fewer than 100 eosinophils per cubic millimeter of blood before injection are discarded.

EVALUATION

The percentage decrease of eosinophil blood count after 3 h is averaged per group. From dose–response curves, potency ratios are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The test is relatively simple and has the advantage of requiring little material.

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N.2.1.2.4

Liver Glycogen Test in Rats

PURPOSE AND RATIONALE

The liver glycogen deposition test, as described by Stafford et al. (1955), is a simple and specific test for glucocorticoid activity (Dorfman 1962; Ringler 1964; Silber and Arcese 1964).

PROCEDURE

Male Sprague–Dawley rats weighing 140–160 g are adrenalectomized. They are fed stock laboratory diet and 1% sodium chloride solution. On the morning of the fourth postoperative day, food is withdrawn. On the morning of the fifth day, the drinking fluid is withdrawn and the rats are given the test compound by a single subcutaneous injection in appropriate dosages suspended in 0.5 ml sesame oil. Seven hours later, the rats are sacrificed. The livers are removed and blotted on filter paper to remove blood, weighed, dropped into

flasks containing 10 ml hot 30% potassium hydroxide and digested on a hot plate. The digest is diluted to 100 ml and a 50-fold dilution of an aliquot is used for analysis.

Then, 10 ml 0.2% anthrone in 95% sulfuric acid is slowly added to 5 ml of liver digest dilution with cooling. The mixture is heated in a boiling water bath for 10 min and then placed into cold water. Optical density is measured in a spectrophotometer at 620 µm using the anthrone-reagent as blank. Calibration curves are established using glucose as standard. Groups of five animals are used for each dose and for the vehicle controls. Three doses of test compound and of standard are used to find dose–response activities. Standard doses of hydrocortisone are 0.5, 1.0, and 2.0 mg per animal subcutaneously or 1.25, 2.5, and 5.0 mg per animal orally.

EVALUATION

The amount of glycogen expressed as glucose is calculated per gram liver weight. Dose–response curves are established for each compound and for the standard in order to calculate potency ratios.

MODIFICATIONS OF THE METHOD

The method can be used to determine the time course and duration of action of derivatives such as corticosteroid esters compared to the free alcohol (Vogel 1963, 1965) and for evaluation of topical and systemic activity (Alpermann et al. 1982).

The liver glycogen deposition in the adrenalectomized mouse has been used by Dorfman et al. (1946) and by Venning et al. (1946) to evaluate glucocorticoid activity. Liver tryptophan peroxidase activity in the rat is decreased after adrenalectomy and increased after administration of corticosteroids (Knox and Auerbach 1955) indicating an early effect of corticoids.

The enzyme activity of tryptophan pyrrolase in guinea pig liver is increased by systemic administration of corticoids, but this effect is short-lived and achieved only with high subcutaneous doses (Albrecht et al. 1979). In contrast, the enzyme is markedly reduced by protracted application of corticoids to the skin (“dermatocorticoids”).

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N.2.1.2.5

Anti-Inflammatory Activity of Corticoid Hormones

GENERAL CONSIDERATIONS

Most of the tests described in the Sect. H.3 (anti-inflammatory activity) have been used for evaluation of corticosteroids.

The effects on acute inflammation are less suitable, [Ultraviolet erythema in guinea pigs (see H.3.2.2.1), Vascular permeability in rats (see H.3.2.2.2), Paw edema in rats (see H.3.2.2.6)], whereas the methods measuring subacute inflammation are very well suited [Granuloma pouch test in rats (see H.3.2.2.8), Cotton granuloma test in rats (see H.3.2.3.1), Glass rod granuloma test in rats (see H.3.2.3.3), Sponge implantation technique (see H.3.2.3.2)].

The molecular mechanisms of the anti-inflammatory actions of glucocorticoids were reviewed by Barnes (1998). Development of dissociated steroids which are more active in transrepression (interaction with transcription factors) than transactivation (binding to glucocorticoid response elements) as reported by Vayssiere et al. (1997) was recommended.

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N.2.1.2.6

Animal Studies for Corticoid Hormone Evaluation

Several examples are presented here for the specific pharmacology of corticoids. The methods applied in some instances are the same methods as for antiphlogistic and anti-inflammatory compounds. Corticosteroids intended for systemic treatment or local application are administered daily for 3–14 days, and the pharmacological effects are determined. NMRI mice (26–28 g) or Wistar rats (140–160 g) are housed according to institutional guidelines with access to food and water *ad libitum*; 8–11 animals may be randomly allocated to the different treatment groups.

Croton Oil-Induced Ear Inflammation

This test is performed by local application, to reduce or prevent the effect of a local irritant. Topical application of the nonspecific contact irritant croton oil, a mixture of several phorbol esters, leads to acute inflammation characterized by edema and a mainly granulocytic cell infiltration into the skin. Swelling of the ear due to inflammation is prevented by the corticoid solution or suspension. For topical application, corticosteroids are dissolved in the same vehicle as used for croton oil and may be applied separately, or together with the irritant croton oil.

Systemic application of corticosteroids (subcutaneously) may also be performed 2 h before croton oil application. At the maximum of the inflammatory reaction, animals are killed, and ears (mice, area $\approx 1 \text{ cm}^2$) or a punch biopsy (rats, 10-mm diameter) of each ear are weighed as an indicator of edema formation. The tissue samples are then snap-frozen in liquid nitrogen in polypropylene tubes and kept at -20°C for up to 24 h (Tonelli et al. 1965; Alpermann et al. 1982; Vayssiere et al. 1997).

Peroxidase Activity Assay

Peroxidase activity, as a measure for total granulocyte infiltration, may be assayed in the tissue samples by using a modification of a method described by Schotelius et al. (2002).

Induction of Tyrosine Aminotransferase (TAT) in Vivo

In a similar manner as performed *in vitro*, the induction of tyrosine aminotransferase (TAT) may be investigated *ex vivo* in tissue of treated animals. TAT in-

duction is evaluated 6 h after compound administration (subcutaneously) to juvenile rats by determination of TAT activity in liver homogenates. Animals are killed, and biopsy samples (10-mm diameter) are taken from the liver and snap-frozen. Biopsy samples are homogenized in 2 ml of homogenizing buffer (140 mM KCl in 20 mM KPO₄ buffer, pH 7.6) and centrifuged at 24,000 *g* for 20 min at 4°C. Supernatants are assayed for protein content by the BCA (bicinchoninic acid) Protein Assay Kit from Pierce. Then 20 µl of supernatant diluted 1:50 in PBS is incubated for 30 min at 37°C with 200 µl of TAT-reaction buffer (tyrosine 1.2 mg/ml or 6.6 mM/45 mM KH₂PO₄/0.06 mM pyridoxal-5'-phosphate/12 mM oxoglutaric acid, adjusted to pH 7.6 with KOH). After stopping the reaction with 10 M KOH and further incubation for 30 min, extinction was measured at 340 nm, and TAT activity is calculated in relation to 500 µg/ml total protein content. TAT induction is defined as *x*-fold increase in TAT activity measured as OD at 340 nm in comparison to the TAT activity in vehicle-treated animals (Thompson et al. 1966).

ACTH Suppression in Rats

Animals are housed under conditions of minimum stress before the study, in a quiet laboratory room. Six hours after application of compounds, animals are killed with minimum stress (decapitation in a separate room), and EDTA-anticoagulated blood is collected from the abdominal aorta. Plasma ACTH content is determined using the ACTH ¹²⁵I-assay system (ImmuChem Double Antibody hACTH) following the manufacturer's instructions (ICN).

The method requires considerable experience because the isolation of animals and sacrificing with a minimum of stress are difficult to achieve. Refer to pretreatment with lower doses of dexamethasone to improve this test method. Treatment with increasing doses of the corticosteroids and prednisolone as the reference standard will reduce the ACTH concentrations, such tests however have an inherently high variability of the vehicle-treated control groups (Sakakura et al. 1981; Baumann et al. 1985).

Increased Blood Glucose in Rats (Hyperglycemia Induced by Corticoids)

Animals are isolated before the study to avoid stress and fasted for 16 h. Six hours after application of compounds, animals are killed, and EDTA-treated blood is collected from the abdominal aorta to assess the corticoid-induced rise in blood glucose. Glucose in plasma is measured by colorimetric serum glucose determi-

nation by using hexokinase and glucose-6-phosphate dehydrogenase with a Hitachi 904 automatic analyzer (Roche Diagnostics).

Skin Atrophy After Chronic Corticoid Application

To assess the effect of local application on collagen composition of the skin, the test compound or the reference compound prednisolone (75 µl on a marked area of 9 cm² in 95% ethanol/5% isopropyl myristate) was applied daily for 19 days to the dorsal skin of nude rats (strain hr-hr, 120–140 g, Iffa-Credo). Animals were killed on day 20. Skin thickness was determined by using a specifically designed dial thickness gauge. Mean values were derived by measuring two adjacent treated skin areas. To determine skin-breaking strength of treated skin, a dorsal skin patch (5 × 5 cm) was removed. The skin patch was placed on filter paper, and two double-T-piece skin strips (50 mm long, 4 mm wide at the narrowest point) were punched in a caudal–cranial direction out of the patch. The skin strips were covered with moistened filter paper to avoid drying and were fixed with the wider ends into an apparatus developed in-house to measure skin-breaking strength. At a constant rate of stretch (200 mm/min), the force necessary to tear the skin strip was determined with a pressure sensor and was expressed as the skin-breaking strength (Alpermann et al. 1982; Vogel and Petri 1985).

Effect of Chronic Treatment on Thymus, Spleen, Adrenal, and Body Weight

To determine effects of repeated-dose corticoid treatment on body weight, animals were weighed before and after completion of treatment. Thymus, spleen, and adrenal glands were removed from animals killed after systemic or topical treatment and weighed. Organ weights are expressed as absolute values or as a percentage inhibition when compared with untreated animals (Laschet et al. 1960; Stoeck et al. 2004).

EVALUATION

For the examples given here, group means were calculated and the statistical significance of the effects of TAT, glucose induction, and ACTH suppression was assessed by using Dunnett's test. Differences between test compound and the corresponding prednisolone group (used as the reference standard) were assessed by using the Mann–Whitney test.

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N.2.1.3

Effects of Steroids on Mechanical Properties of Connective Tissue

N.2.1.3.1

Breaking Strength of Bones

PURPOSE AND RATIONALE

Patients with Cushing's disease or after long-term treatment with corticosteroids have an increased susceptibility for bone fractures. The related animal tests were developed as a model for corticosteroid-induced osteoporosis, and they are of historical interest. The tests are based on changes in the mechanical properties of bone. Surprisingly, in chickens as well as in rats, short-term treatment with low and medium doses of glucocorticosteroids increased the breaking strength of bones dose-dependently whereas very high doses induced a decrease of breaking strength.

PROCEDURE

White Leghorn chicken at an age of 14 days weighing 170 ± 20 g were used. The animals were treated

for 14 days by intramuscular injection of 0.2 up to 100 mg/kg cortisol (reference compound) or equivalent doses of other glucocorticoids in oily solution. At autopsy, body weight and comb weight are recorded and the femur and tibia bones from both sites are weighed. Length of bone (l) and outer diameter (D) of the diaphysis are measured using calipers. The bone is fastened on both ends, supported on edges 5 mm distant from the ends, and broken in the central portion of the diaphysis by a special device attached to an Instron-instrument. Breaking load (P) is recorded. The contralateral bone is cut at the middle of the diaphyseal shaft into rings by a small saw. Inner (d) and outer diameter (D) are measured in two perpendicular directions.

EVALUATION

Breaking strength (σ) of the hollow bones is calculated according to the technical formula for hollow cylinders:

$$\sigma = \frac{8}{\pi} \times \frac{P \times l \times D}{D^4 - d^4}.$$

Doses between 0.2 and 2.0 mg/kg cortisol and 0.05 and 1.0 mg/kg prednisolone show a parallel increase of breaking strength allowing the calculation of potency ratios. With higher doses, the response changes to inhibition of breaking strength. Excessive doses of 50 and 100 mg/kg cortisol, which are in the range of the LD₅₀, induce a decrease of breaking strength (Vogel 1968, 1969, 1990; Vogel and Ther 1962).

MODIFICATIONS OF THE METHOD

In young and adult rats, 10 days of treatment with cortisol and other corticosteroids induce a dose-dependent increase of breaking strength, whereas with longer treatment (30 days) a bell-shaped dose-response (increase with low doses, decrease with high doses) is seen.

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N.2.1.3.2**Tensile Strength of Femoral Epiphyseal Cartilage in Rats****PURPOSE AND RATIONALE**

The epiphyseal cartilage of rats is very sensitive to treatment with hormones and other desmotropic drugs. The tensile strength of the distal femoral epiphyseal plate is decreased after adrenalectomy and is restored or increased by treatment with low doses of corticosteroids.

PROCEDURE

Male Sprague-Dawley rats weighing 120 ± 10 g are treated with several doses of corticosteroids or other hormones for periods of between 1 and 14 days. After sacrifice, the hind legs are exarticulated in the hip joint and fastened at the collum femoris. Longitudinal tension always results in rupture of the distal femoral epiphyseal cartilage (Ther et al. 1963; Vogel and Ther 1964; Vogel 1969). The ultimate load of the femoral epiphyseal plate is recorded by an Instron instrument at an extension rate of 5 cm/min.

Single injections of doses between 10 and 100 mg/kg cortisol acetate or 2.5 and 40 mg/kg prednisolone acetate induce a dose-dependent increase of tensile strength up to twice the control levels after 24–48 h, which subsides after 96 h. Repeated administration up to 15 days also results in a dose-dependent increase. There is a maturation-dependent increase of tensile strength in untreated rats.

The effect of other hormones, e.g., gonadal steroids, on cartilage is much smaller than that of glucocorticosteroids.

EVALUATION

Dose–response curves for steroids can be established allowing the calculation of potency ratios in experiments with single-dose or repeated-dose administration.

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N.2.1.3.3**Tensile Strength of Tail Tendons in Rats****PURPOSE AND RATIONALE**

The thread-like tail tendons of rats are very suitable for studying the mechanical properties of connective tissue. They are easy to prepare and consist predominantly of collagen. Like other organs of connective tissue, such as bone, cartilage, and skin, the tensile strength of tail tendons is reduced after adrenalectomy and dose-dependently increased after short-term treatment with glucocorticosteroids (Vogel 1965, 1969).

PROCEDURE

Male Sprague-Dawley rats weighing 120 ± 10 g are treated with increasing doses of corticosteroids or other hormones for periods of between 1 and 14 days. After sacrifice, the tail is amputated at its base. The tail skin and the last few coccygeal vertebrae are removed. Single tendons are pulled out from the dorsal and ventral bundles and kept in saline solution. Tendons of the same diameter (0.25 mm) are selected with a stereomicroscope. Since the diameter of the tendons may be influenced by long-term treatment of the animals, tendons of the same vertebral insertion are tested, alternatively. For this purpose, the 10th vertebra is counted from the tail tip. All six tendons (four on the ventral and two on the dorsal side) inserting on this vertebra are removed and tested. The tendons are fixed in special clamps at a distance of 2.0 cm and immersed in physiological saline. Stress–strain curves and ultimate loads are determined with an Instron instrument with an extension rate of 5 cm/min.

A single injection of cortisol acetate (10–100 mg/kg) or prednisolone acetate (2 and 20 mg/kg) causes a dose-dependent increase of tensile strength allowing the calculation of a potency ratio of 1:4 for prednisolone versus cortisol. Repeated injections of cortisol acetate in doses between 1.0 and 50 mg/kg (s.c.) or prednisolone acetate in doses between 0.05 and 50 mg/kg (s.c.) show the same dose dependence and potency ratio. The tensile strength of tail tendons is similarly changed by treatment with gonadal steroids, depending on the dose and duration of treatment, but to a much lesser degree than by corticosteroids.

EVALUATION

For changes in mechanical properties, the potency ratios of glucocorticoids versus cortisol as the standard are calculated.

MODIFICATIONS OF THE METHOD

In addition to tensile strength, other biophysical parameters can be determined in tail tendons, such as hysteresis behavior (Vogel 1984). The ratio of energy input versus energy dissipation is calculated. Furthermore, dependence on strain rate and relaxation behavior under the influence of corticosteroids (Vogel 1989) as well as retardation (strain rate under constant load) have been studied (Vogel and Schorning 1990).

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N.2.1.3.4**Tensile Strength of Skin Strips in Rats****PURPOSE AND RATIONALE**

Clinical experience shows a decrease of skin thickness after long-term systemic or local treatment with corticosteroids (skin atrophy, thinning). The mechanical properties of human skin after treatment with corticosteroids are difficult to measure. As with other connective tissues, in animal experiments a clear increase of tensile strength of skin has been found after short-term administration of corticosteroids (Vogel 1969, 1971a, 1971b, 1974, 1981, 1986, 1993b).

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated with increasing doses of corticosteroids or other substances for periods of between 1 and 14 days. After sacrifice, the back of the animals is shaved and a flap of skin measuring about 5×5 cm is removed. The skin flap is placed between two pieces of plastic material with known thickness. By these means, the actual thickness of the excised skin is measured by calipers. Two dumbbell-shaped specimens are cut with a special punch in perpendicular direction to the body axis, having in the middle a width of 4 mm. They

are fixed between the clamps of an Instron instrument. In the usual experiments, stress–strain curves and ultimate loads are recorded at a strain rate of 5 cm/min. From stress–strain curves, the values of ultimate load and ultimate extension are recorded. The stress–strain curves are almost linear in part, allowing the calculation of the modulus of elasticity. Tensile strength is calculated from ultimate load divided by original cross-sectional area.

Dose–response curves for 10 and 100 mg/kg cortisone acetate or 2 and 20 mg/kg prednisolone acetate show a sharp increase of ultimate load and tensile strength up to 5 days of treatment, a continuous decrease of skin thickness upon longer treatment, and a decrease of ultimate load up to 2 months of treatment, which is below controls for ultimate load, but not for tensile strength (Vogel 1970a).

EVALUATION

Dose–response curves are established for corticosteroids versus the standard cortisone and potency ratios with confidence limits are calculated.

MODIFICATIONS OF THE METHOD

With a similar method, the tensile strength of skin wounds after treatment with corticosteroids was studied in rats (Vogel 1970a, 1970b). See also Sect. P.11.4.

Strain rate influences the values of ultimate load, tensile strength and modulus of elasticity, but not the effect of corticosteroids (Vogel 1972).

In contrast to parameters indicating the strength and elasticity of collagen, the parameters indicating plasticity, such as relaxation, hysteresis, strain at constant load (creep experiments), and isorheological behavior, are less dramatically changed by corticosteroids but indicate a decrease of viscosity or an increase of stiffness.

For a more detailed description of relaxation experiments (Vogel 1973) see Sect. P.11.2.1.5; of hysteresis experiments (Vogel 1976, 1989), see Sect. P.11.2.1.6; of creep experiments (strain at constant load) (Vogel 1977, 1989), see Sect. P.11.2.1.8; of measurement of isorheological behavior (Vogel 1987, 1989), see Sect. P.11.2.1.7; and for *in vivo* experiments in rat skin (Vogel and Denkel 1985; Vogel 1989, 1993a), see Sect. P.11.3.

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N.2.1.4

Topical Effects of Glucocorticosteroids on Skin

N.2.1.4.1

Skin Thickness and Tensile Strength

PURPOSE AND RATIONALE

From clinical experience it is known that a decrease of skin thickness occurs after long-term local treatment with corticosteroids. The mechanical properties of human skin after treatment with corticosteroids are dif-

ficult to assess. A clear increase of tensile strength of skin after short-term local administration of corticosteroids has been found in animal experiments, whereas skin thickness decreased (Alpermann et al. 1982; Vogel and Petri 1985).

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 150 ± 10 g are used. The animals are shaved before and once again during treatment. The animals are treated once daily over 10 days with various concentrations of the test compound or the standard or with fixed concentrations of the test compound in different galenical preparations. The test material is applied in a volume of 0.2 ml to an area of 4 cm^2 to the shaven back skin. The animals are kept in individual cages to avoid systemic absorption by mutual licking. Two days after the last treatment the animals are sacrificed, a flap of skin measuring about 5×5 cm is removed, and skin thickness is determined. The skin flap is placed between two pieces of plastic material with known thickness. The thickness of the excised skin is measured by calipers. Perpendicular to the body axis two dumbbell-shaped specimens are punched out. Stress-strain curves are recorded using an Instron instrument. The following parameters are determined:

- load at rupture (ultimate load)
- extension at rupture (ultimate strain)
- skin thickness
- tensile strength (ultimate load divided by cross-sectional area)
- modulus of elasticity (calculated from the straight part of the stress-strain curve).

EVALUATION

Dose-response curves are established for the parameters ultimate load, ultimate strain, skin thickness, tensile strength, and modulus of elasticity for the standard (prednisolone acetate) and the test compound allowing the calculation of potency ratios with confidence limits.

CRITICAL ASSESSMENT OF THE METHOD

In addition to corticosteroid-induced atrophy of the skin after topical application assessed by histology, mechanical parameters can be determined.

MODIFICATIONS OF THE METHOD

Kapp et al. (1977) used specially devised apparatus for rats covering the site of substance application, which

guarantees an exclusive dermal absorption and excludes oral ingestion of the steroid.

In addition to skin thickness and breaking strength, Töpert et al. (1990) determined thymus weight, water and glycosaminoglycan content in the skin of rats after topical application of steroids over 30 days.

Iwasaki et al. (1995) measured the skin atrophy in Wistar rats by locally applied clobetasol-17-propionate, a synthetic glucocorticoid, and the influence of simultaneously applied RU 486. Then, 24 h after the last application, the thickness of the skin was measured with a dial skin thickness gauge, with an accuracy of 0.01 mm. The treated skin was pinched lengthwise, and both skin surfaces were held vertically between two plastic discs of the gauge.

Hartop et al. (1978) measured transepithelial water loss (TEWL) in the skin of rats deficient in essential fatty acids (Prottey et al. 1976; Lowe and Stoughton 1977) after local treatment with corticosteroids.

Woodbury and Kligman (1992) recommended the hairless mouse as a model for assaying the atrophogenicity of topical steroids. Epidermal atrophy was determined by the number of cell layers of viable epidermis on five fields under $\times 400$ magnification. Dermal thickness was determined under $\times 400$ magnification. The total number of sebocytes in all visualizable sebaceous glands was counted in ten fields at $\times 250$ magnification.

Van den Hoven et al. (1991) used the hairless mouse as a model to distinguish between local and systemic atrophogenic effects of topical steroids. Male hairless mice (strain h/h-NMRI) were treated on the left flank with test preparations once daily for 3 weeks. During this time, body weight was measured and the skin-fold thickness of treated and untreated sides was determined using a graduated micrometer. On day 21, the animals were sacrificed and dermal thymidine uptake and weights of the thymus were determined.

Corticosteroid-induced skin atrophy in hairless mice can be prevented by tretinoin (Lesnik et al. 1989; Schwartz et al. 1994). In these studies, albino Skh-hairless-1 mice were treated topically twice daily with corticosteroids or in combination with tretinoin. At the end of the treatment, all mice were sacrificed and specimens of the dorsal skin were frozen for light microscopy and quantification of glycosaminoglycans, fibronectin, and collagen.

Wrench (1980) applied commercially available topical corticosteroid preparations to the proximal halves of albino mouse tail for 21 days and measured epidermal thickness by histology. All steroids caused epidermal thinning, except clobetasone butyrate.

Altmeyer and Buhles (1981) tested the anti-acanthogenic effect of topically applied steroids after long-term treatment of guinea pigs (up to 118 days) by histological measurement of epithelial thickness. For delimitation of the measuring lines, the upper boundary was taken as that between the stratum granulosum and the stratum corneum, and the lower boundary as that between the basal cell layer and the corium.

Kajita et al. (1986) tested epidermal beta-adrenergic adenylate cyclase responses in pigs after topical application of glucocorticoids. A significant increase of this receptor response was observed 24 h following topical application of potent glucocorticoid ointments. Domestic white-haired pigs were anesthetized with 30 mg/kg nembutal i.p. Four 5×5 cm areas were chosen and the following treatments were administered to each area: (1) topical administration of glucocorticoid ointments; (2) UVB irradiation alone (230 mJ/cm^2); (3) topical application of glucocorticoid ointments following UVB irradiation; (4) no treatment as a control. After 24 h the treated pigs were anesthetized again and skin specimens were obtained from the four areas by means of a Castroviejo keratome (Storz Instrument, St. Louis, Mo., USA) adjusted to the 0.3 mm setting. Each skin slice obtained by the Castroviejo keratome was cut into 5×5 cm squares, which were washed 3 times in RPMI 1640 medium and preincubated in RPMI 1640 medium for 37°C to standardize the cAMP level (Yoshikawa et al. 1975). After the preincubation, two pieces of skin squares were randomly selected and were incubated with various adenylate cyclase stimulators (Iizuka et al. 1985). The concentrations of epinephrine, adenosine, and histamine added to the incubation medium were $50 \mu\text{M}$, 2 mM and 1 mM , respectively. The cAMP content in skin squares was measured by radioimmunoassay using a Yamasa cAMP assay kit (Yamasa Shoyu, Tokyo, Japan) after partial purification (Yoshikawa et al. 1975). The cAMP phosphodiesterase activities in skin squares were measured by the method of Adachi et al. (1976) who purified multiple forms of pig epidermal cyclic nucleotide phosphodiesterases by DEAE-cellulose column chromatography.

In vitro experiments measuring the effects of retinoids and glucocorticoids on the beta-adrenergic adenylate cyclase system of pig skin epidermis were performed by Iizuka et al. (1985).

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N.2.1.4.2

Assay of Topical Glucocorticoid Activity in Transgenic Mice

PURPOSE AND RATIONALE

Katchman et al. (1995) proposed a transgenic mouse model as a biological assay of topical glucocorticosteroid potency.

PROCEDURE

A homozygous line of transgenic mice expressing 5.2 kilobases (kb) of the human elastin promoter region linked to the chloramphenicol acetyltransferase (CAT) reporter gene was developed (Hsu-Wong et al. 1994). For this purpose, 5.2 kb of human elastin 5'-flanking DNA is linked to a 0.7-kb CAT gene, followed by 0.3 kb of DNA with a polyadenylation signal. This linearized construct is injected into fertilized oocytes, and a line of transgenic mice expressing the human elastin promoter, as detected by CAT activity, is developed. These transgenic mice have no clinical phenotype and they do not express human elastin protein, as no part of the coding sequence is contained within the transgene. The human elastin promoter/CAT construct is expressed in a tissue-specific and developmentally regulated manner.

Test animals are 4- or 5-day-old hairless pups, homozygous for the transgene. In each experiment, pups of the same litter are used for comparison between glucocorticosteroid-treated and control preparations in parallel. Steroid preparations are tested by applying 0.03 g uniformly on their dorsal surface ($\approx 14 \text{ mg/cm}^2$). Control animals receive the same amount of cream base. The test animals are separated from each other and from their mothers and are sacrificed at different time points. Skin biopsy samples are removed immediately from the treated area.

For the CAT assay, skin is homogenized with a Polytron tissue homogenizer in 0.25 M TRIS hydrochloride (pH 7.5). The homogenates are freeze-thawed three times and centrifuged at 10,000 g for 15 min. The protein content of the supernatant is determined by a commercial kit and aliquots, containing 100 mg of protein, are assayed for CAT activity in the linear range of the assay (Gorman et al. 1982) (see Sect. N.2.1.1.2).

EVALUATION

The significance of differences between treatment groups is evaluated by Student's *t*-test.

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N.2.1.4.3**Effect on Epidermal DNA Synthesis****PURPOSE AND RATIONALE**

Du Vivier et al. (1978), Marshall and Du Vivier (1978), Marshall et al. (1981), and Clement et al. (1983) evaluated the local and systemic effects of topically applied corticosteroids on epidermal DNA synthesis in hairless mice.

PROCEDURE

Hairless mice of either sex, 2–4 months old, are dosed in groups of six with test preparations or control base: 0.03 ml of test drug formulation is spread over 3 cm² of the anterior dorsal skin. The posterior half of the dorsal surface of the animal is left untreated. Animals are injected subcutaneously with 25 mCi (methyl-³H) thymidine, specific activity 5 Ci/mM, in the right thigh 1 h before being sacrificed 6 or 24 h after application of test formulation.

The neck and back skin samples, with at least 2 cm unsampled skin left between them (to avoid inclusion of treated skin in the back sample) are removed. The epidermis is separated from the dermis by placing the sample, dermis downwards, on a stainless steel plate at 57°C for 25 s. The epidermis can then easily be separated from the dermis with a scalpel blade. The epidermal samples are wrapped in aluminum foil and stored at 20°C until analysis.

Approximately 30 mg of epidermis in 5 ml of a 0.24 M sodium phosphate buffer solution, containing 8 M urea, 1% sodium lauryl sulfate and 1 mM EDTA at pH 6.8, is lysed using an ultrasonic disintegrator. DNA is extracted from the cell lysate by column chromatography using hydroxylapatite. The epidermal cell lysate is added to the hydroxylapatite column and washed with a buffer containing 0.24 M sodium phosphate and 8 M urea at pH 6.8 to remove RNA and protein. The urea is then removed with 50 ml of 0.14 M sodium phosphate buffer at pH 6.8. Double-stranded DNA, which binds to hydroxylapatite under low-salt

buffer conditions, is eluted with 0.48 M sodium phosphate buffer at pH 6.8, and a 2-ml sample is collected.

The DNA concentration in the sample is determined by UV absorption at 260 nm in a spectrophotometer. Then 0.5-ml aliquots of the sample are added to 12 ml scintillation cocktail and 0.2 ml N HCl in scintillation vials. Each sample is prepared in triplicate and counted for 20 min/vial in a scintillation counter.

EVALUATION

The results are initially expressed as mean counts per minute (c.p.m.) for the vial triplets, converted to mean disintegrations per minute (d.p.m.) and the results expressed as d.p.m./μg DNA. The significance of differences between treatment groups is evaluated by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Marks et al. (1973) described a method for the assay of topical corticosteroids in mice based on changes in thymidine incorporation after treatment. Sticky tape was applied five or six times to the dorsal skin of mature male hairless mice of the Harwell strain to remove the horny layer. A similar quantity of topical test preparations (about 70 mg) was applied on the stripped dorsal skin of each mouse. A dressing of cotton gauze lined with a plastic film was applied and secured with strapping, staying for periods of from 5 to 156 h. Then 4 h before sacrifice the mice were intraperitoneally injected with 0.15 ml of a 0.1% Colcemid solution together with 30 μCi of tritiated thymidine. The dorsal skin area previously treated was removed, fixed in 10% buffered formalin and stained with hematoxylin and eosin. For estimation of the mitotic index, cells in the prophase, anaphase, and metaphase stages were counted. To quantitate the labeling index, the number of labeled basal and suprabasal cells was estimated as a proportion of the total number of basal cells.

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N.2.1.4.4

Induction of Drug-Metabolizing Enzymes

PURPOSE AND RATIONALE

Finnen et al. (1984, 1985) evaluated the effects of topical application of glucocorticosteroids on the activity of drug-metabolizing enzymes in the skin of adult hairless mice. The ability of steroid preparations to induce enzyme activity was related to their clinical potency.

PROCEDURE

Adult male and female hairless mice are treated with 0.3 g of test creams or cream base applied to the dorsal skin and rubbed in until no visible traces remain. Then 16–18 h after treatment, the animals are sacrificed, the treated area of skin removed, and placed epidermis-down on a curved surface, and subcutaneous fat and muscle are removed using a scalpel blade. Epidermis and dermis are separated by a heating technique (Thompson and Slaga 1976). The tissue is then placed in 0.1 M phosphate buffer (pH 7.4) and minced finely using surgical scissors. Scissors-minced tissue is then homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) using a Polytron homogenizer. Whole-skin homogenates are then centrifuged at 9000 g for 20 min and the resulting supernatant used as the source for the determination of 7-ethoxycoumarin O-deethylase (Greenlee and Poland 1978). For the determination of ethoxyresorufin O-dealkylation (Pohl and Fouts 1980) the 9000 g supernatants are further centrifuged at 100,000 g for 1 h, the resulting microsomal pellet resuspended in 0.1 M phosphate buffer (pH 7.4), and used as the enzyme source. The DNA content of the 9000 g pellet is estimated by the diphenylamine reaction (Burton 1956).

EVALUATION

The significance of differences between treatment groups is evaluated by Student's *t*-test.

REFERENCES AND FURTHER READING

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N.2.1.4.5

Cornea Inflammation in Rabbits

PURPOSE AND RATIONALE

Leibowitz and Kupferman (1974), Leibowitz et al. (1978, 1992) and Cantrill et al. (1975) studied the anti-inflammatory efficacy of topical corticosteroids for treatment of glaucoma in rabbits. Again, this test is purely of historical interest.

PROCEDURE

New Zealand albino rabbits weighing 1.8–2.3 kg are anesthetized for approximately 5 min with 15 mg/kg i.v. thiamylal sodium. A corneal inflammatory response is produced by intralaminar inoculation of 0.03 ml of laboratory-grade clove oil. Before the induction of corneal inflammation, the rabbits are given three intravenous inoculations of 1.85×10^6 Bq/kg of an aqueous solution of tritiated thymidine (24.79×10^{10} Bq/mol) at 24-h intervals. The intracorneal injection of clove oil is given concomitantly with the third thymidine injection, and 24 h later, therapy is initiated. A standard drop (0.05 ml) of drug is instilled hourly for a total of six doses, and then, after a lapse of 18 h, one drop is administered hourly for an additional total of seven doses.

Corticosteroid preparations are tested in different concentrations. A control group is run with each experimental trial; control rabbits are handled in the same manner as experimental rabbits, except that the control rabbits receive either prednisolone acetate (positive control) or no treatment (negative control). At autopsy, a 10-mm penetrating corneal button is removed by trephination, tissue samples are placed in a commercially available solubilizing agent (Soluene 350, Packard Instrument), 1 ml per cornea, until dissolved. The samples are counted for a minimum of 10 min, measuring the amount of radioactivity in each cornea sample.

EVALUATION

The significance of differences between treatment groups is evaluated by Student's *t*-test.

REFERENCES AND FURTHER READING

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N.2.1.4.6**Endotoxin-Induced Uveitis in Rats****PURPOSE AND RATIONALE**

Salmonella endotoxin, a lipopolysaccharide, produces uveitis 12–24 h after injection into the footpad of rats. Uveitis is characterized by miosis, iris hyperemia, increase of protein content in aqueous humor, and inflammatory cell accumulation in the anterior uvea and aqueous humor. Glucocorticoids strongly inhibit endotoxin-induced uveitis by suppressing inflammatory mediators (Cousins et al. 1982; Tsuji et al. 1997).

PROCEDURE

Female inbred Lewis rats weighing about 160 g are used. Then 500 µg/kg *Salmonella* endotoxin dissolved in saline is injected into the footpads. Twelve hours later, the animals are sacrificed and both eyes of each animal used in the experiments. The anterior chamber of the eye is punctured, using a 27-gauge needle, to collect the aqueous humor. Then 5-µl aqueous humor samples are placed into 495 µl of phosphate-buffered saline containing 1% paraformaldehyde. The number of cells in the aqueous humor is then counted using a flow cytometry system. The cell number for both eyes of each animal is averaged for statistical analysis of the results.

In topical applications, glucocorticoids are instilled (5 µl/eye) three times at 1 h before and 3 and 7 h after lipopolysaccharide injection. For systemic application, glucocorticoids are injected subcutaneously 3 h after lipopolysaccharide injection.

Total RNA is isolated from the iris-ciliary body with a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). Each RNA sample is extracted from the pooled iris-ciliary bodies of both eyes of each animal. The extracted RNA is quantified after which 4 µg is used for DNA synthesis via polymerase chain reaction.

EVALUATION

Dunnett's multiple comparison test was applied for statistical analysis.

Further methods for measuring local or topical activity of corticosteroids are very suitable for evaluating local corticosteroid activity, described in Sect. H.3 "Anti-inflammatory activity", such as oxazolone-induced ear edema in mice (see Sect. H.3.2.2.4) and croton-oil-induced ear edema in rats or mice (see Sect. H.3.2.2.5).

Furthermore, methods described in Chap. I "Anti-arthrotic and immunomodulatory activity", Sect. I.2.2.4 "Passive cutaneous anaphylaxis", and Sect. I.2.2.6 "Delayed type hypersensitivity are recommended as models for testing topical corticosteroid potency".

REFERENCES AND FURTHER READING

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N.2.1.4.7**Croton Oil-Induced Ear Inflammation**

Topical application of the nonspecific contact irritant croton oil, a mixture of several phorbol esters, leads to acute inflammation characterized by edema and a mainly granulocytic cell infiltration into the skin. For topical application, compounds are dissolved in the same vehicle as used for croton oil and are co-applied. Systemic application of compounds (s.c.) is performed 2 h before croton oil application. At the maximum of the inflammatory reaction, animals are killed, and ears (mice, area ≈ 1 cm²) or a punch biopsy (rats, 10 mm diameter) are weighed as an indicator of edema formation, then snap-frozen in liquid nitrogen in polypropylene tubes and kept at –20°C for up to 24 h.

N.2.1.5**Anti-Glucocorticoid Activity****N.2.1.5.1****Adrenal and Thymus Involution****PURPOSE AND RATIONALE**

The involution of thymus and adrenal glands induced by hydrocortisone can be antagonized by compounds with anti-glucocorticoid activity (Dorfmann 1962).

PROCEDURE

Groups of six to ten immature male Sprague-Dawley rats weighing 60–70 are injected subcutaneously daily for 6 days with 0.2 ml of the antagonistic test compound in 0.5% aqueous carboxymethylcellulose solution. Hydrocortisone acetate is used as the agonist in daily doses of 0.2 mg per animal. Controls receive hydrocortisone acetate only. On the seventh day, the animals are sacrificed and the adrenal and thymus weights determined.

EVALUATION

The relative weight [quotient of adrenal or thymus weight (mg) and body weight (g)] is calculated for each rat. Means of the antagonist-treated groups are compared with the means of hydrocortisone-only controls. An increase of thymus and adrenal weight relative to the hydrocortisone control indicates anti-glucocorticoid activity.

MODIFICATIONS OF THE METHOD

Inhibition of tyrosine aminotransferase induced by corticosterone or dexamethasone was used by Vincent et al. (1997) to characterize the anti-glucocorticoid properties of a novel synthetic steroid.

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N.2.2**Mineralocorticoid Activity****N.2.2.1****In Vivo Methods****N.2.2.1.1****General Considerations**

Very early bioassays (“Survival tests”) have been used to standardize extracts of adrenal cortex for mineralocorticoid activity (Grollman 1941; Ringler 1964; Tolksdorf et al. 1956). For evaluation of mineralocorticoid activity these tests have been replaced by bioassays measuring electrolyte excretion in the urine.

Several animal species do not survive after adrenalectomy. However, they can be kept alive by administration of mineralocorticoids. The first animal species used successfully was the adrenalectomized drake (Bülbring 1937). The methods employing adrenalectomized rats and mice (Dorfman 1962) suffered from the fact that in these species aberrant adrenal cortical tissue may occur, which is not removed by surgical adrenalectomy. In contrast, male Syrian golden hamsters weighing 50–60 g are very suitable for testing adrenocortical activity (Junkmann 1955).

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N.2.2.1.2**Electrolyte Excretion****PURPOSE AND RATIONALE**

Mineralocorticosteroids enhance sodium retention and potassium excretion. The sodium excretion in adrenalectomized rats is dose-dependently decreased. This parameter can be used to assess the mineralocorticoid activity of test compounds (Kagawa et al. 1952; Marcus et al. 1952).

PROCEDURE

Male Sprague-Dawley rats weighing 140–160 g are adrenalectomized. They are maintained on 1% sodium chloride solution. On the morning of the fourth post-operative day, food and drinking fluid are withdrawn. On the following day, each rat is given 5 ml water by stomach tube; 1 h later 5 ml of 0.9% sodium chloride solution is given orally. Test compounds are injected s.c. in 0.2 ml of vehicle suspension. Desoxycorticosterone acetate (DOCA; 1–40 μ g per rat) is used as standard (reference compound). The rats are placed in metabolic cages, two rats per cage, three cages per dosage group, for 4 h, and then removed from the cages. The use of light, transient ether anesthesia in this method is obsolete. Urine volume is recorded and

in addition cages may be rinsed with a distilled water spray. Appropriate dilutions of urine are analyzed for sodium with a flame photometer. The amount of sodium excreted by each rat is calculated, and group means are expressed as percentage of excretion by control animals.

EVALUATION

Percent reduction of sodium excretion compared with controls is calculated for each dosage group. Potency ratios are calculated with reference to desoxycorticosterone acetate.

MODIFICATIONS OF THE METHOD

Simpson and Tait (1952) measured both urinary sodium and potassium and used the sodium-to-potassium ratio as an index of the electrolyte activity of corticoids. Nikisch et al. (1991) infused glucocorticoid-substituted adrenalectomized rats with saline-glucose solution containing aldosterone and measured sodium and potassium concentrations in 1-h fractions of urine. The anti-aldosterone activity was assessed by the ability of test compounds to reverse the aldosterone effect on the urinary Na/K ratio.

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N.2.2.2

In Vitro Methods

N.2.2.2.1

Mineralocorticoid Receptor Binding

PURPOSE AND RATIONALE

Rat kidney receptor preparations and radioactively labeled aldosterone were used to test affinity for the min-

eralocorticoid receptor (Raynaud et al. 1975, 1979; Pasqualini and Sumida 1977; Ojasoo and Raynaud 1978; Wambach and Higgins 1971).

PROCEDURE

Kidney homogenates of adrenalectomized rats are centrifuged at 0°C for 10 min at 800 g in a buffer solution (10 mM Tris, 0.25 M saccharose, HCl, pH 7.4). Following addition of RU 28362 at 0.001 mM (to inhibit binding of aldosterone to the glucocorticoid receptor) the supernatant is centrifuged again at 105,000 g for 60 min. The supernatant (cytosol) is removed and incubated at 0°C with ^3H -aldosterone (5 nM) and increasing concentrations of test compounds (0–25,000 nM). Nonspecific binding is determined in the presence of 1 μM aldosterone. Free ^3H -aldosterone is removed from the incubation medium by charcoal-dextran absorption after 1 h or 24 h of incubation (Raynaud 1978). Following centrifugation, the concentration of receptor-bound ligand is determined in the supernatant by liquid scintillation counting.

EVALUATION

The following parameters are calculated: total binding of ^3H -aldosterone, nonspecific binding in the presence of 1 μM aldosterone, specific binding (= total binding–non-specific binding), % inhibition with specific binding as a percentage of the control value.

For structure–activity studies, all compounds are first tested at a single high concentration (25,000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is determined using seven to eight different concentrations of compound. The binding potency of compounds is expressed as binding affinity (RBA), relative to the standard compound (aldosterone).

MODIFICATIONS OF THE METHOD

Affinity for the mineralocorticoid receptor was tested using the cytosol of rabbit kidneys (Claire et al. 1993) or rat kidney slices (Funder et al. 1974). It is also useful to use overexpressed human mineralocorticoid receptors.

Monkey kidney COS-1 cells were transfected with plasmids containing the human mineralocorticoid receptor – cloned by Arriza et al. (1987) – and the glucocorticoid receptor for binding studies with various steroids (Rupprecht et al. 1993a, 1993b).

A survey of mineralocorticoid receptor ligands has been given by Sutano and de Kloet (1991).

Specific bioluminescent *in vitro* assays for selecting potential anti-mineralocorticoids were developed by Jausons-Loffreda et al. (1994).

Wehling (1994) found evidence for a membrane-bound mineralocorticoid receptor in human mononuclear leukocytes.

The human mineralocorticoid receptor was used by Grassy et al. (1997) to study structure–activity relationships of steroids with anti-mineralocorticoid activity.

Davioud et al. (1996) described the synthesis and biological activities of new steroidal diazoketones as potential photoaffinity labeling reagents for the mineralocorticoid receptor.

Fagart et al. (1997a) recommended [³H-2]-21-diazoprogesterone as a potent photoaffinity labeling reagent for the mineralocorticoid receptor.

Fagart et al. (1997b) proposed a three-dimensional model for antagonism in the human mineralocorticoid receptor.

The biological and clinical relevance of glucocorticoid and mineralocorticoid receptors has been reviewed by Funder (1997).

Rogerson et al. (1999, 2003) studied the structural determinants of aldosterone-binding selectivity in the mineralocorticoid receptor. To characterize ligand-binding specificity, chimeras were made between the human mineralocorticoid receptor and glucocorticoid receptor ligand-binding domains. Models of the mineralocorticoid receptor ligand-binding domain bound to aldosterone and spironolactone were created based on the crystal structure of the progesterone receptor ligand-binding domain. These techniques have been used by Rogerson et al. (2004) to evaluate the differences in the determinants of eplerenone, spironolactone, and aldosterone binding to the mineralocorticoid receptor.

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N.2.2.2.2**Transactivation Assay for Mineralocorticoids****PURPOSE AND RATIONALE**

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows determination of the agonistic and also of the antagonistic activity of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

PROCEDURE**Vector Construct**

The expression plasmid pRShMR containing the full-length coding sequence of the mineralocorticoid receptor expressed from the long terminal repeat of the Rous sarcoma virus is prepared according to Arriza et al. (1987). The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

CV-1 cells and COS-1 cells for transient infections are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 4 mmol/l L-glutamine, penicillin and streptomycin. Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80% confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 μ g pRShMR (hMR expression plasmid) and 5 μ g pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 μ g Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin reagent dilutions are combined in

a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml of DMEM supplemented with 10% fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3% charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. As negative control for the reporter gene induction, cells are cultured with 1% ethanol. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay (Chloramphenicol Acetyltransferase)

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37°C water bath) three times. Protein concentrations in the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

EVALUATION

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to demonstrate the potency of the test hormone. Aldosterone (10^{-10} to 10^{-6} mol/l) serves as standard.

For anti-mineralocorticoid activity, CAT activity in the presence of 10^{-8} mol/l aldosterone is set as 100% and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the antihormone.

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2.

MODIFICATIONS OF THE METHOD

White et al. (1994) described a simple and sensitive high-throughput assay for steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector, which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human

cell lines. In the cervical carcinoma cell line HeLa, which expresses high levels of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone. These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase assay.

Rupprecht et al. (1993a, 1993b) examined the functional agonistic and antagonistic activity of several steroids by co-transfecting human mineralocorticoid or human glucocorticoid receptor expression vectors, together with a mouse mammary tumor virus-luciferase (MTV-LUC) reporter gene into the human neuroblastoma cell line SK-N-MC. Transfections were performed using an electroporation system (Biotechnologies and Experimental Research, San Diego, Calif., USA).

Lombès et al. (1994) used a co-transfection assay in CV-1 cells to study the discrimination of aldosterone from natural and synthetic glucocorticoids by the human mineralocorticoid receptor. Cells were transfected by the calcium phosphate method with pRShMR, a plasmid that contains the entire coding sequence of the human mineralocorticoid receptor; pFC31Luc, which contains the mouse mammary tumor virus (MMTV) promoter driving the luciferase gene; pCH110 encoding the β -galactosidase as an internal transfection control; and pSP72 as plasmid carrier.

Lim-Tio et al. (1997) studied the determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor in three cell lines: CV-1 cells; a porcine renal epithelial cell line, LLC-PK1; a pig kidney cell strain, and RN33B; a neuronal medullary raphe cell line. The reporter gene used was MMTV-LUC (the long terminal repeat of the mouse mammary tumor virus promoter linked to the luciferase reporter gene). RSV-CAT (Rous sarcoma virus promoter-chloramphenicol acetyl transferase gene) was used as an internal control for the transfection efficacy.

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N.2.2.3

Anti-Mineralocorticoid Activity

N.2.2.3.1

Electrolyte Excretion

PURPOSE AND RATIONALE

The method is based on the mineralocorticoid activity of desoxycorticosterone acetate (agonists) (reference compound) reversed by spironolactone (reference antagonist) and other anti-mineralocorticoids to be evaluated (test compounds).

PROCEDURE

Male Sprague-Dawley rats weighing 140–160 g are adrenalectomized and maintained on 1% NaCl solution as drinking fluid. On the morning of the fourth postoperative day, food and drinking fluid are withdrawn. On the following day, each rat is given 5 ml water by stomach tube; 1 h later it is given 5 ml 0.9% NaCl orally and injected with 40 µg desoxycorticosterone acetate. At a separate site, the antagonistic test compound is injected s.c. in 0.2 ml of vehicle suspension. Spironolactone, 50 µg or 500 µg, is injected as the reference compound with anti-mineralocorticoid activity. Rats are placed in metabolic cages, two rats per cage, three cages per dosage group, for 4 h of urine collection. Urine volume is recorded and cages rinsed over the collection cylinders with a distilled water spray. Appropriate dilutions of collected urine are analyzed for sodium and potassium with a flame photometer.

EVALUATION

The amount of water, sodium, and potassium excreted per 100 g bodyweight is calculated. The product of water volume and sodium excretion is divided by the potassium excretion. The excretion ratios (quotient) are compared for untreated adrenalectomized rats, animals treated with DOCA, and animals treated with spironolactone

MODIFICATIONS OF THE METHOD

Anti-mineralocorticoid activity of spironolactone and its analogs has been tested in adrenalectomized golden hamsters treated simultaneously with daily injections desoxycorticosterone acetate over a period of 3 weeks (Vogel, unpublished data 1965). The mean survival of adrenalectomized animals of 4.7 days was prolonged to 13.5 days by daily subcutaneous injections of 2 mg desoxycorticosterone acetate. Additional injection of 0.5 or 1.0 mg spironolactone reduced the survival time.

Losert et al. (1985) tested the ability of several steroids with progestogenic potency to inhibit the renal actions of aldosterone in adrenalectomized, glucocorticoid-treated rats. The rats were continuously infused with an isotonic solution of low sodium content (0.05% NaCl + 5.2% glucose, 3 ml/rat per hour) supplemented with D-aldosterone (1 µg/kg per h) resulting in a long-lasting reduction in renal sodium excretion, an increase in renal potassium excretion, and hence a decrease in the urinary Na/K ratio. The test drugs were administered either subcutaneously or orally 1 h before the start of infusion. The anti-mineralocorticoid activity was judged by the increase in the aldosterone-

lowered Na/K ratio in urine collected at hourly intervals up to 21 h.

Cutler et al. (1978, 1979) described a potent mineralocorticoid receptor antagonist with decreased antiandrogenic activity relative to spironolactone.

De Gasparo et al. (1987) evaluated epoxy-spironolactone derivatives for their anti-mineralocorticoid activity (Kagawa test) and their antiandrogenic and progestogenic side-effects *in vitro* and *in vivo*.

Gómez-Sánchez et al. (1990) reported the effect of intracerebroventricular infusion of mineralocorticoid antagonists on the hypertension in rats produced by chronic subcutaneous administration of aldosterone.

The evolution of aldosterone antagonists has been reviewed by Garthwaite and McMahon (2004) and Hu et al. (2005) featuring eplerenone, a molecule with improved steroid receptor selectivity and pharmacokinetic properties in humans compared with spironolactone.

The beneficial effects of eplerenone have been demonstrated in rats with myocardial infarction (Masson et al. 2004; Fraccarollo et al. 2005) and in rats with autoimmune myocarditis (Wahed et al. 2005) as well as in mice with chronic heart failure (Wang et al. 2004).

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N.3 Ovarian Hormones

N.3.0.1 Ovariectomy of Rats

PROCEDURE

Ovariectomy is performed in immature female rats weighing 40–60 g (Bomskov 1939; Emmens 1969; May 1971). Animals are anesthetized. A single transverse incision is made in the skin of the back. This incision can be shifted readily from one side to the other by traction on the skin. A small puncture is then made over the site of the ovary, which can be seen through the abdominal wall, embedded in a pad of fat. The top of a pair of fine forceps is introduced and the fat around the ovary is grasped, care being taken not to rupture the capsule around the ovary. The tip of the uterine horn is crushed with a pair of artery forceps and the ovary, together with the fallopian tube, is removed with a single cut by a pair of fine scissors. Usually, no bleeding is observed. In older rats, the tip of the uterine horn may be ligated and the ovary removed distally from the ligature. The ovary of the other side is removed in

the same way. The skin wound is closed by one or two clips. The animal recovers immediately.

Ovariectomized rats are now obtained from commercial suppliers when needed, the surgical procedure however is easy to learn, and licenses for animal experimentation are readily obtained for qualified laboratory personnel.

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N.3.1 Estrogens

N.3.1.1 In Vitro Methods

N.3.1.1.1 Estrogen Receptor Binding

PURPOSE AND RATIONALE

The nuclear receptor assays are used to estimate the estrogenic activity of test compounds. Estradiol-17 β is used as the reference compound. Estrogen receptors are prepared from mouse uteri or from human endometrium. Measurements of association rates and dissociation rates at different temperatures allow evaluation of relative binding affinities (Bouton and Raynaud 1977, 1978). Binding to the cytosolic and the nuclear fractions is measured.

PROCEDURE

Cytosol Preparation

Uteri from 18-day-old female Swiss mice are removed and homogenized at 0°C in 1:50 (w/v) of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose buffer in a conical homogenizer. Human endometrium from menopausal women is frozen within 2 h of hysterectomy and stored in liquid nitrogen until use. The frozen endometrium is pulverized and homogenized in 1:5 (w/v) Tris-sucrose buffer. Homogenates are centrifuged for 1 h at 105,000 g.

Dextran-Coated Charcoal (DCC) Adsorption Technique

Binding is measured as follows. A 100- μ l aliquot of incubated cytosol is stirred for 10 min at 0°C in a microtiter plate with 100 μ l of Dextran-coated charcoal

(DCC) suspension (0.625% Dextran 80000, 1.25% charcoal Norit A) and then centrifuged for 10 min at 800g. The concentration of bound steroid is determined by measuring the radioactivity in a 100- μ l aliquot of supernatant.

Determination of Specific Binding in Mouse Uterus Cytosol as a Function of Steroid Concentration, Incubation Time and Temperature

Triplicate aliquots of 125 μ l of cytosol are incubated with 5 or 25 nM labeled steroid (estradiol-17 β) either for 2 or 24 h at 0°C or for 2 or 5 h at 25°C in the absence (total binding) or presence (nonspecific binding) of a 100-fold excess of radio-inert steroid. Bound steroid is measured by DCC adsorption.

Measurement of Association Rate at 0°C

For this, 1 or 5 nM concentration of labeled steroid is added to cytosol maintained at 0°C. Every 5 min for 1 h after addition of the labeled steroid, a 100- μ l aliquot is transferred into a 5000 nM radio-inert steroid solution in a microtiter plate to stop the reaction. Bound radioactivity is determined by DCC adsorption.

Measurement of Dissociation Rate at 25°C

Dissociation rate is measured by the isotopic dilution technique. Radio-inert steroid (2500 nM) is added to crude cytosol previously incubated with 5 nM labeled steroid for 15 h at 0°C. After different times of incubation at 25°C, 100- μ l samples are treated with DCC at 0°C in order to determine bound radioactivity. Specific binding is evaluated by subtracting nonspecific binding from total binding.

Nuclear Uptake

Homogenate samples (0.5 ml; two uteri per 0.5 ml) are incubated with 2.5, 5 or 25 nM labeled steroid for 5 or 60 min at 25°C, then cooled on ice and centrifuged at 800g for 10 min at 0°C. The pellet (crude nuclei) is washed 3 times with 1 ml Tris-sucrose buffer, dissolved in 0.5 ml Soluene (Packard) and counted.

Nuclear Extract Preparation

After incubation with 25 nM labeled steroid for 1 h at 25°C, the crude nuclei are resuspended in 0.5 ml Tris-sucrose buffer, to which 50 μ l of 4 M KCl is added. The suspension is stirred (vortex), left for 30 min at 0°C and then centrifuged for 30 min at 105,000g. The radioactivity in a 100- μ l supernatant sample (nuclear extract) is counted.

EVALUATION

Relative binding affinity is calculated from the percentage of radioligand bound in the presence of competitor compared to radioligand bound in its absence, plotted against the concentration of unlabeled competing steroid. A standard curve for the competition of the unlabeled radioligand is constructed with the use of nine to ten concentrations; five or six concentrations of the competitor are tested. These are chosen to provide a linear portion of the semilog plot which crosses the point of 50% competition. From this plot, the molar concentration of unlabeled radioligand or steroid competitor is determined that reduces radioligand binding by 50%.

The relative affinity of a test compound is the ratio of unlabeled radioligand concentration to competitor concentration, at 50% competition. This ratio is multiplied by 100.

Association rate (k_{+1}) is calculated by the slope of the line

$$k_{+1}t = (2.3/E_0 - R_0) \log(E R_0/R E_0)$$

where E_0 and E represent free steroid and R_0 and R free receptor at time $t=0$ and time t , respectively.

Dissociation rate (k_{-1}) is calculated from the slope of the line

$$k_{-1} = -2.3 \log B/B_0$$

where B_0 and B represent bound steroid at time $t=0$ and time t , respectively.

Similar studies were reported by: Raynaud et al. 1975; Clark et al. 1976; Katzenellenbogen et al. 1977; Ojasoo and Raynaud 1978; Clark et al. 1982; Mukawa et al. 1988; Smanik et al. 1989; Labrie et al. 1990; Chander et al. 1991; Dhar et al. 1994.

MODIFICATIONS OF THE METHOD

Sheep uteri were used for the preparation of cytosol by Shutt and Cox (1972).

Pons et al. (1990) described receptor binding of estrogens and anti-estrogens in the estrogen-receptor-positive breast cancer cell line MCF-7, using the firefly luciferase assay (Brasier et al. 1989) as endpoint.

Ludwig et al. (1990) described a microliter well assay for quantitative measurement of estrogen receptor binding to estrogen-responsive elements.

Hwang et al. (1992) studied the use of tetrahydrochrysenes, inherently fluorescent, which are high-affinity ligands for the estrogen receptor.

Estrogen receptor binding has also been used to study the mechanism of action of nonsteroidal anti-es-

trogens (Jordan et al. 1977; Wakeling and Slater 1980; Astroff and Safe 1988).

The sequence and expression of human estrogen receptor complementary DNA was reported by Greene et al. (1986).

Solution structure of the DNA-binding domain of the estrogen receptor has been described by Schwabe et al. (1990).

Obourn et al. (1993) studied the hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor overproduced in insect cells using a baculovirus expression system.

Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β are reported by Tremblay et al. (1997).

Nichols et al. (1998) showed that agonists and antagonists differently position the C-terminus of the ligand-binding domain (helix 12) and the F domain of the estrogen receptor.

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N.3.1.1.2

Transactivation Assay for Estrogens

PURPOSE AND RATIONALE

Transient transfection of a suitable cell culture with a reporter gene controlled by an estrogen-responsive element (ERE) has become a convenient assay for estrogens.

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone-responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988).

The assay in the estrogen-receptor-positive human breast cancer cell line MCF-7, described by Meyer et al. (1994) as a rapid luciferase transfection assay for transcription activation of estrogenic drugs, has been used for evaluation of synthetic estrogen antagonists by Von Angerer et al. (1994, 1995) and Biberger and Von Angerer (1996).

PROCEDURE

MCF-7 cells used for transfection are grown in Dulbecco's Modified Essential Medium supplemented with 10% fetal calf serum, 100 U penicillin, 100 µg streptomycin and 150 mg L-glutamine in 500 ml of medium without phenol red. Shortly before confluence, the cells are washed with 10 ml of PBS. Cells are gently shaken for a few seconds with trypsin-EDTA solution (4 ml) and after removal of the solution incubated for 2 min at 37°C. After addition of 10 ml of medium, the cell suspension (0.5 ml/well) is transferred to 6-well plates containing 2 ml of medium. Cells are grown until the density of the monolayer is about 50% (1–2 days) before 2 µg of the luciferase reporter plasmid EREwtc luc, harboring the luciferase gene from *Photinus pyralis*, is added. For a successful transfection, it is necessary to generate a very fine precipitate of the DNA by subsequent dilution with 45% water, 5% M CaCl₂, and 50% HBS buffer and continuous shaking. After 20 min at room temperature, an opalescent solution should be obtained.

After addition of the DNA solution, the medium is removed and the cells are washed with 2 ml of PBS, followed by treatment with glycerol (15% in PBS) for 2 min. After washing with PBS, fresh medium containing the test substances is added. The maximum of luciferase expression is reached 18 h after addition of the transfection solution. At this time, the medium is removed and cells are washed with PBS. Cell lysis and quantification of luminescence is performed according to the procedure described in the luciferase assay system E1500 of PROMEGA (Serva, Heidelberg, Germany). Luminescence is measured in a luminometer Lumat LB (Berthold, Wildbad, Germany) as relative

light units, which are converted into fg luciferase by a calibration curve.

EVALUATION

Dose–response curves of luciferase activity after induction by the test substances and estradiol as control (10^{-14} to 10^{-9} M) are established.

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2. Variation in transfection efficacy is the major cause of data variation and needs to be controlled for valid interpretation of data.

MODIFICATIONS OF THE METHOD

With the same method as for MCF-7 cells, Von Angerer et al. (1994) and Biberger and Von Angerer (1996) transfected HeLa-cells using the estrogen expression receptors HE0, HEG0, HE15 and HEG19, together with the receptor plasmid EREwtc luc.

Bergmann et al. (1994) determined estrogenic activity by a transient transfection assay in CHO cells deficient in estrogen receptors (ER) transfected with an expression vector encoding the ER using an estrogen-responsive reporter gene construct, (ERE)₂-TATA-CAT, containing two estrogen-response elements linked to a TATA promoter and the chloramphenicol transferase reporter gene.

For assessing environmental chemicals for estrogenicity, Shelby et al. (1996) recommended a transcriptional activation assay in ER-transfected HeLa cells using the estrogen-responsive reporter, ERE81CAT, and the pRSV vector containing the mouse ER cDNA without the neomycin resistance cassette. Triplicate samples for each hormone concentration were harvested at 28 h post-transfection and assayed for CAT protein using the CAT-ELISA kit (Boehringer Mannheim).

Several authors used yeast for assays of estrogenicity (McDonnell et al. 1991; Pierrat et al. 1992; Kohno et al. 1994; Bush et al. 1996; Tran et al. 1996; Odum et al. 1997).

Pierrat et al. (1992) constructed yeast strains in which the *Saccharomyces cerevisiae* URA3 gene is induced by the human estrogen receptor. Promoter sequences required for both basal and activated transcription of URA3 were replaced with estrogen-response elements positioned upstream of the native TATA box. These constructs were integrated at the TRP1 locus of a yeast strain in which the natural URA3 gene has been deleted and the integrants were transformed with shuttle plasmids expressing wild-type or truncated derivatives of human estrogen recep-

tor. Transformants were assayed for growth on uracil-deficient medium plus or minus estradiol, for resistance to 5-fluoroorotic acid, and for activity of orotidine-5'-monophosphate decarboxylase, the product of the URA3 gene.

Tran et al. (1996) used the yeast strain ER(wt) expressing human estrogen receptor and an estrogen-sensitive reporter to characterize the estrogenic or anti-estrogenic activities of polynuclear aromatic hydrocarbons.

Gaido et al. (1997) described a yeast-based steroid hormone receptor gene transcription assay to evaluate estrogenic and androgenic activity. The yeasts contain two separate plasmids: (1) an expression plasmid which contains the CUP1 metallothionein promoter fused to the human estrogen receptor cDNA, and (2) a receptor plasmid carrying two estrogen-response elements or a reporter plasmid carrying two copies of a progesterone/androgen-responsive element upstream of the structural gene for β -galactosidase.

Chu et al. (2004) described transrepression of estrogen receptor β signaling by nuclear factor- κ B in ovarian granulosa cells.

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N.3.1.1.3

Estrogen-Dependent Cell Proliferation

PURPOSE AND RATIONALE

Specific cell proliferation induced by estrogens is the principle of these assays. Some human breast cancer cell lines, such as MCF-7 and T47-D cells, respond to estrogens with proliferation (Zondek 1935; Eisler 1964). This effect has been used for assays of estrogenic and antiestrogenic activity (Miller and Katzenellenbogen 1983; Scholl et al. 1983; Thompson et al. 1984; Palkowitz et al. 1997; Zacharewski 1997).

PROCEDURE

MCF-7 breast adenocarcinoma cells are maintained in MEM (Minimal Essential Medium), minus phenol red (which is estrogenic at high concentrations) supplemented with 10% fetal bovine serum (FBS), 2 μ M L-glutamine, 1 μ M sodium pyruvate, 10 μ M HEPES, nonessential amino acids and 1 mg/ml bovine insulin. Ten days prior to assay, MCF-7 cells are switched to maintenance medium supplemented with 10% dextran-coated FBS in place of 10% FBS to deplete internal stores of steroids. MCF-7 cells are

removed from maintenance flasks using cell dissociation medium [$\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS (phenol red-free) supplemented with $10\ \mu\text{M}$ HEPES and $2\ \text{nM}$ EDTA]. Cells are washed twice with assay medium and adjusted to 80,000 cells/ml. Approximately $100\ \mu\text{l}$ (8000 cells) is added to flat-bottomed microculture wells and incubated at 37°C in a 5% CO_2 humidified incubator for 48 h to allow for cell adherence and equilibration transfer. Serial dilutions of test compounds, or dimethylsulfoxide (DMSO) as a diluent control, are prepared in assay medium and $50\ \mu\text{l}$ transferred to triplicate microcultures, followed by $50\ \mu\text{l}$ assay medium for a final volume of $200\ \mu\text{l}$. After an additional 48 h incubation, microcultures are pulsed with $1\ \mu\text{Ci}$ [^3H]thymidine (specific activity $6.7\ \text{Ci}/\text{mmol}$) for the last 4–6 h of culture and the assay terminated by freezing at -70°C . Microcultures are then thawed and harvested using a Skatron semiautomatic cell harvester. Samples are counted by liquid scintillation using a Wallace BetaPlate β -counter.

EVALUATION

Plotting of dpm versus compound concentration is used to determine the half-maximal effective concentrations EC_{50} or inhibitory concentration IC_{50} .

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N.3.1.2

In Vivo Methods

N.3.1.2.1

Vaginal Cornification Assay

PURPOSE AND RATIONALE

This is an early bioassay for estrogenic activity based on epithelial proliferation. The Allen-Doisy test for

vaginal cornification in rodents (Allen and Doisy 1923) is based on the observations of Stockard and Papanicolaou (1917), who first reported the cyclic cornification of the vaginal epithelium in guinea pigs.

PROCEDURE

Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. They are kept for about 1 week on standard laboratory diet and water ad libitum. The test compounds are administered orally or subcutaneously in 0.5% solution of carboxymethylcellulose or in cotton seed oil injected at several doses to groups of 10–20 rats. Doses of 0.02, 0.1, and 0.5 μg estradiol per animal are used as standard. The compounds are dosed twice daily, for example, on two following days at 10:00 a.m. and 5:00 p.m. At 5:00 p.m. of the third day and at 10:00 a.m. of the fourth day vaginal smears are taken using cotton swabs moistened with saline. The smears are transferred to a glass slide and stained for 10 min with 5% aqueous methylene blue solution. They are evaluated microscopically according to the following scores:

- 0 diestrus smear, mainly leukocytes, few epithelia cells
- 1 mixture of leukocytes and epithelial cells
- 2 proestrus smear, nucleated or nucleated plus cornified cells
- 3 estrus smear, cornified cells only.

Only animals showing a score of 2 or 3 of cornification are considered to be positive.

EVALUATION

The number of positive animals in each dosage group is recorded. ED_{50} values are calculated and potency ratios compared with the standard estradiol may be determined.

MODIFICATIONS OF THE METHOD

The sensitivity of the assay is increased by local vaginal application of estrogens in ovariectomized rats or mice (Emmens 1969).

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N.3.1.2.2

Uterine Weight Assay

PURPOSE AND RATIONALE

This is an early bioassay for estrogenic activity based on myometrial/endometrial proliferation. Repeated administration of estrogens induces a dose-dependent increase of uterine weight in ovariectomized rats.

PROCEDURE

Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. Groups of five to ten animals are injected daily with several doses of the test compound or the standard (estradiol 0.03 to 0.06 µg per animal s.c.) for 7 days. The test compound is administered orally or subcutaneously in 0.5% solution of carboxymethylcellulose or in cotton seed oil. Controls receive the vehicle. On the 8th day, the animals are sacrificed and uterine weights determined.

EVALUATION

Dose–response curves are established and potency ratios are calculated, using at least two doses of test compound and oestradiol standard.

Similar studies were reported by: Zondek 1935; Junkmann 1957; Emmens 1969; Nishino et al. 1991; Van de Velde et al. 1994)

MODIFICATIONS OF THE METHOD

Rubin et al. (1951) used albino mice 23–25 days of age weighing approximately 8 g. The mice are given subcutaneous injections once daily for 3 days of an oil solution of the hormone. Then, 24 h after the last injection, the animals are sacrificed and uterine and body weights recorded. The uterine ratio is calculated by dividing uterine weight in milligrams by body weight in grams, multiplied by 100 (relative uterine weight). Two groups receive a standard preparation and two groups the unknown.

Bhakoo and Katzenellenbogen (1977) showed that one of the earliest biosynthetic tissue responses after estrogen binding in the rat uterus, the synthesis of

a specific uterine protein called “induced protein”, is antagonized by progesterone.

In addition to uterus weight, Branham et al. (1993) studied luminal and glandular epithelium height in cross-sections of the uterine horns of rats by histomorphology.

Uterine peroxidase activity was proposed as a marker for estrogen action by Lyttle and DeSombre (1977) and Astroff and Safe (1991).

Odum et al. (1997) compared the rodent uterotrophic assay with a yeast estrogenicity assay.

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N.3.1.2.3

Chick Oviduct Method

PURPOSE AND RATIONALE

This is an early bioassay for estrogenic activity based on proliferation of the oviduct. The weight of the oviduct of young chickens is increased dose depend-

dently by natural and synthetic estrogens (Tullner and Hertz 1956; Lerner et al. 1958; Dorfmann 1969).

PROCEDURE

Seven-day-old pullet chicks are injected subcutaneously twice daily with solutions of the test compound in various doses for 6 days. Doses between 0.02 and 0.5 µg estradiol-17β per animal serve as standard. Six to ten chicks are used for each dosage group. On the day after the last injection, the animals are sacrificed and the weights of the body and oviduct determined.

EVALUATION

The ratio oviduct weight/body weight is calculated for each animal (relative weight). Mean values are plotted as dose–response curves in order to calculate potency ratios.

MODIFICATION OF THE METHOD

The assay was used for evaluation of anti-estrogenic activity using simultaneous injection of 0.6 µg estradiol-17β and the inhibitor, e. g., progestagens, and calculation of the percentage of oviduct weight in estrogen/anti-estrogen-treated animals versus the estrogen control group.

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N.3.1.3

Anti-Estrogenic Activity

N.3.1.3.1

Antagonism of Estrogen Effect on Uterus Weight

PURPOSE AND RATIONALE

These assays are modifications of estrogen bioassays. The effect of estrogen treatment on uterine weight in ovariectomized rats was antagonized by anti-estrogenic compounds.

PROCEDURE

Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. Groups of five to ten animals are injected daily for 7 days with estradiol 0.03

to 0.06 µg per animal s.c. and various doses of the test compound or estradiol alone. The test compound is administered in 0.5% solution of carboxymethylcellulose or in cotton seed oil either orally or injected subcutaneously. On day 8, the weight of the uterus is determined and the inhibitory effect of anti-estrogenic compounds is assessed.

EVALUATION

The anti-estrogenic effect is expressed as a percentage reduction of estrogen-stimulated uterine weight by test compounds.

Similar studies were reported by: Byrnes and Shipley 1955; Dorfmann 1969; Terenius 1971; Wakeling and Bowler 1988; Levesque et al. 1991; Kangas 1992; Nique et al. 1994)

MODIFICATIONS OF THE METHOD

Three-week old female NMRI mice can be used for determination of uterus weight (Lerner et al. 1958).

The inhibition by anti-estrogens of vaginal cornification induced by estradiol can be used as the assessment parameter.

The chick oviduct weight can be adapted for the assay of anti-estrogenic activity (Tullner and Hertz 1956).

Anti-estrogenic activity may also be determined in castrated immature male rats. The animals show an increase in seminal vesicle weight when treated with 20 µg estradiol for 5 days, and the effect is counteracted by anti-estrogenic steroids (Byrnes et al. 1953).

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N.3.1.3.2

Aromatase Inhibition

PURPOSE AND RATIONALE

Estrogen synthesis by local action of the enzyme aromatase occurs in various tissues, and is also found in estrogen-dependent tumors (Brodie 1992). Specific aromatase (estrogen synthetase) inhibitors are potential therapeutic agent for estrogen-dependent tumors, e.g., some breast cancers. 4-Hydroxyandrostendione (4-OHA) inhibits aromatase completely but also causes destruction of the enzyme (irreversible inhibitor). Test compounds can be evaluated both *in vitro* (Häusler et al. 1989) and *in vivo* (Geelen et al. 1991). Tests are based on stimulation of estrogen biosynthesis by luteinizing hormone (LH) and inhibition of this effect by aromatase inhibitors.

PROCEDURE

For *in vitro* experiments, ovarian tissue from adult golden hamsters (*Mesocricetus auratus*) is used. Estrus cycle is monitored for at least three consecutive 4-day estrus cycles prior to the experiment. The experiments for evaluating inhibitor effects are performed with ovaries obtained from animals sacrificed on day 4 (proestrus), at the time of preferred estrogen synthesis. The ovaries are excised, freed from adhering fat tissue, and quartered. The quarters are transferred to plastic incubation flasks with 2 ml of Krebs-Ringer-bicarbonate (KBR) salt solution, pH 7.6, containing 8.4 mM glucose. The flasks are gassed with O₂/CO₂ (95%/5%), tightly closed, and placed in a shaker/water bath (37°C) for incubation of the fragments. The incubation media are replaced with fresh KBR after preincubation for 1 h.

The ovaries are further incubated for 4 h in the presence or absence of ovine LH (100 ng/ml) and inhibitors to be tested. 4-OH-Androstendione is used as standard in concentrations between 0.33 and 330 μ M. At the end of the experiment, the incubation media are removed and centrifuged. In the supernatant, estrogen, progesterone, and testosterone are determined by radioimmunoassay.

EVALUATION

The results are expressed as percentage inhibition of oestradiol synthesis relative to control incubations containing 100 ng/ml LH in the absence of inhibitor. Statistical analyses are performed using Dunnett's *t*-test.

MODIFICATIONS OF THE METHOD

Geelen et al. (1991) determined the *in vivo* aromatase activity of test compounds in hypophysectomized rats treated with the estrogen precursor dehydroepiandrosterone (DHEA) sulfate, using the inhibition of cornification of vaginal epithelium and estradiol levels in plasma as parameters. Furthermore, aromatase activity was determined in homogenized ovaries. To the supernatant, [1β -³H]androst-4-ene-3,17-dione was added. Incubation was performed with a NADPH-generating system for 1 h at 37°C. Incubations were terminated by placing the tubes on ice followed by an extraction with chloroform. After phase separation by centrifugation, the aqueous phase was diluted with an equal volume of dextran-coated charcoal suspension. Following centrifugation, the ³H₂O content was determined by liquid scintillation counting. The amount of ³H₂O is a measure of the amount of estrogen produced.

Zaccheo et al. (1989) studied the antitumor activity of aromatase inhibitors in rats with tumors induced by 7,12-dimethylbenzanthracene (DMBA).

Wouters et al. (1993) studied the inhibition of aromatase activity in FSH-stimulated rat granulosa cells by vorozole, a selective, nonsteroidal aromatase inhibitor.

Suzuki et al. (1996) described changes in prostate volume and histopathological findings in androstenedione-treated castrated beagle dogs as a bioassay for an aromatase inhibitor.

Takahashi et al. (1997) tested the inhibition of aromatase activity in the microsomes from fresh human placenta by the amount of tritiated water released from [1β -³H]androstenedione. For biological proof, the dose-dependent suppression of androstenedione-induced uterine hypertrophy in immature rats was measured.

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N.3.1.3.3

Anti-Estrogenic Effect on MCF-7 Breast Cancer Cells

PURPOSE AND RATIONALE

The MCF-7 cell line, derived from a pleural effusion of a malignant breast cancer, is a widely studied model for hormone-dependent human breast cancer. These cells contain functional estrogen receptors and show a pleiotropic response to estrogen which can be used to evaluate anti-estrogenic effects (Miller and Katzenellenbogen 1983; Scholl et al. 1983; Thompson et al. 1988). The MCF-7 cells proliferate and invade through an artificial basement membrane. Anti-estrogens reduce both the proliferation and invasiveness of MCF-7 cells.

PROCEDURE

MCF-7 cells are maintained in T75 flasks in IMEM (Biofluids, Rockville, Md., USA) supplemented with 2 mM glutamine and 10% fetal bovine serum. To deplete estrogen, the cells are passaged for at least 2 weeks in IMEM supplemented with 5% calf serum which has been treated sequentially with sulfatase and dextran-coated charcoal (DCC) to remove endogenous estrogen.

Estrogen and Anti-Estrogen Treatment

Cells are trypsinized, reseeded in tissue culture dishes (1×10^6 cells/10-cm-diameter Falcon dish), and allowed to adhere overnight in a humidified incubator (37°C, 5% CO₂, 95% air). The cells are treated the next day with either 17 β -estradiol (10^{-9} M) or the anti-estrogen or 0.1% ethanol alone. Four days later, the cells are harvested with trypsin, washed twice in IMEM containing 0.1% BSA, counted with a Coulter

cell counter and tested for chemotaxis and chemoinvasion activities.

Chemoinvasion Assay

Boyden chambers are used (Albini et al. 1987). Polycarbonate filters (12- μ m pore, polyvinylpyrrolidone-free, Nucleopore) are coated with matrigel (25 μ g/filter), a mixture of basement membrane components (Kleinman et al. 1986), which is dried and then reconstituted at 37°C into a solid, even layer over the surface of the filter. Fibroblast-conditioned medium, obtained by incubating confluent NIH-3T3 cells for 24 h with IMEM, is used as the chemoattractant. Cells are harvested with trypsin, washed twice with BSA/IMEM, and added to the top chamber (300,000 cells/chamber). Chambers are incubated in a humidified incubator at 37°C in 5% CO₂ in air for 6, 9, or 12 h. The cells which have traversed the matrigel and attached to the lower surface of the filter are stained with Diff-Quick (American Scientific Products) and quantitated electronically with the Optimax V image analyzer.

Chemotaxis Assay

Chemotaxis assays are performed as described for the chemoinvasion studies with the single exception that the filter surfaces are coated with 5 μ g collagen IV instead of the layer of matrigel. This coats the interstices of the filters but does not form a barrier over the surface. Chemotaxis assays are performed in parallel to the chemoinvasion assays using the same cells and conditioned medium.

EVALUATION

One-way analysis of variance is performed on the data from each experiment.

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N.3.2

Progestional Activity

N.3.2.1

In Vitro Methods

N.3.2.1.1

Gestagen Receptor Binding

PURPOSE AND RATIONALE

Progesterone receptor preparations may be obtained from numerous sources of tissue: uteri from estrogen-primed rabbits (Ojasoo and Raynaud 1978; Boonkasemsanti et al. 1989; Phillips et al. 1990; Cook et al. 1992), castrated-estrogen-treated mice or rats (Philibert and Raynaud 1977; Li et al. 1997), MCF-7 cells derived from human breast tumor (Bergink et al. 1983; Kloosterboer et al. 1988a, 1988b, 1994), breast cancer T47D cells (Meyer et al. 1990), the quail fibroblast cell line QT6 (Schowalter et al. 1991) or human uteri obtained after hysterectomy (Jänne et al. 1976; Pollow et al. 1989a, 1989b, 1992). Tritium-labeled progesterone and R 5020 ([6,7-³H]17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) are used as ligands (Moguilewsky and Raynaud 1979).

PROCEDURE

Relative Binding Affinities

Human uteri obtained after hysterectomy are snap-frozen in liquid nitrogen and stored at -80°C until use. For cytosol preparations, uterine tissues are minced and homogenized with an Ultra-Turrax at $0-4^{\circ}\text{C}$ in ice-cold buffer composed of 10 mM KH_2PO_4 , 10 mM K_2HPO_4 , 1.5 mM EDTA, 3 mM NaN_3 , 10% glycerol, pH 7.5 (PENG buffer). The homogenates are then centrifuged at 105,000 g at 4°C for 30 min. The supernatant is taken as cytosol. The cytosol preparations are incubated with ³H-R 5020 as radioligand at a concentration of 8 nmol/l and increasing concentrations (1×10^{-10} to 1×10^{-5} mol/l) of the competitor steroids overnight at 4°C . Then, unbound steroids are adsorbed by incubating with 0.5 ml of DCC (0.5% Norit A, 0.05% dextran T400 in PENG buffer) for 10 min at 4°C . After centrifugation (10 min at 1500 g at 4°C) 0.5 ml of the supernatant is withdrawn and counted for radioactivity.

Association rate and dissociation rate are determined as described for the estrogen receptor.

EVALUATION

For calculation of **relative binding affinity**, the percentage of radioligand bound in the presence of competitor compared to that bound in its absence is plot-

ted against the concentration of unlabeled steroid. A standard curve for the unlabeled radioligand (progesterone) is constructed with of nine to ten concentrations; five or six concentrations of the competitor are tested. The molar concentrations of unlabeled radioligand and steroid competitors that reduce radioligand binding by 50% are determined. The ratio of unlabeled radioligand and competitor for 50% competition multiplied by 100 is calculated for relative binding affinity.

Association rate (k_{+1}) is calculated by the slope of the line

$$k_{+1}t = (2.3/E_0 - R_0) \log(E R_0 / R E_0)$$

where E_0 and E represent free steroid and R_0 and R free receptor at time $t=0$ and time t , respectively.

Dissociation rate (k_{-1}) is calculated from the slope of the line

$$k_{-1} = -2.3 \log B/B_0$$

where B_0 and B represent bound steroid at time $t=0$ and time t , respectively.

MODIFICATIONS OF THE METHOD

For screening procedures homogenates of rabbit uteri may be used (Philibert et al. 1977).

The binding of the progesterone agonist R 5020 and of the progesterone antagonist RU 486 to the progesterone receptor from calf uterus was characterized by Hurd and Moudgil (1988).

A high-affinity ligand and novel photoaffinity labeling reagent for the progesterone receptor ([³H]DU41165) was described by Pinney et al. (1990).

The different DNA-binding properties of the calf uterine estrogen and progesterone receptors were explained by different dimerization constants (Skafar 1991).

Mutations of the progesterone receptor were found to be responsible for species specificity and have been used for evaluation of agonistic and antagonistic activity (Benhamou et al. 1992; Garcia et al. 1992).

The complete amino acid sequence of the human progesterone receptor has been deduced from cloned cDNA by Misrani et al. (1987).

Structural requirements of the ligand and mapping of the hormone-binding site of the progestin receptor have been discussed by Ojasoo and Raynaud (1990).

Allan et al. (1992) studied conformational changes in the ligand-binding domain induced by various progestins and antiprogestins.

Collins (1994) recommended the ratio between the affinity of a compound to progesterone receptors to the

affinity to androgen receptors as a selection criterion for new oral contraceptives.

Comparative pharmacology of newer progestagens has been reviewed by Kuhl (1996).

Oñate et al. (1994) found that the DNA-binding protein HMG-1 enhances progesterone receptor binding to its target DNA sequences.

The concept of two categories of progestin antagonists based on differences in how they interact with and inactivate the progestin receptor has been discussed by Edwards et al. (1995).

Carbajo et al. (1996) studied the binding of [³H]progesterone to the human progesterone receptor existing in two isoforms hPR-A and hPR-B which differ in that hPR-A lacks 164 amino acids at the amino terminus of hPR-B.

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N.3.2.1.2

Transactivation Assay for Gestagens

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor acts with hormone-responsive elements of hormone-regulated genes, thereby inducing a cascade of transcriptional events (Green and Chambon 1988).

Transactivation assays were used by several authors to test the progestational and antiprogestational activity of steroidal and nonsteroidal compounds (Sobek et al. 1994; Pathirana et al. 1995; Fuhrmann et al. 1996; Jones et al. 1996; Dijkema et al. 1998; Edwards et al. 1998; Schoonen et al. 1998; Zhi et al. 1998).

PROCEDURE

CV-1 cells (African green monkey kidney fibroblasts) are grown in Dulbecco's modified Eagle medium

containing 10% charcoal resin-stripped fetal bovine serum, 2 mM glutamine, and 55 $\mu\text{g}/\text{ml}$ gentamicin. Cells are maintained in an environment of 4% carbon dioxide and routinely passaged from T-255 flasks to 96-well microtiter plates (1.5×10^5 cells/well, 70% confluent) 1 day before transfection.

Cells are transiently transfected by the standard calcium phosphate co-precipitation procedure with 50 ng/well of plasmids coding for: human progesterone receptor-B (hPR-B1) and pRS- β -Gal under constitutive control and a reporter (MTV-LUC) containing a response element for the progesterone receptor. After 6 h, the medium is replaced with medium containing progesterone at concentrations between 10^{-11} and 10^{-5} M or another progestagen. After a 40-h incubation, wells are washed with phosphate-buffered saline and the cells are lysed with Triton X-100-based buffer. An aliquot of lysate (20 μl) is then transferred to Dynatech 96-well plates containing 1.6 mM ATP. LUC activity (chemiluminescence upon addition of luciferin substrate) is determined using a Dynatech ML1000 luminometer, according to the equation:

$$\text{LUC units} = \text{relative LUC unit} \times 10^4.$$

β -Gal activity is determined from the remaining lysate in the original 96-well plates. The substrate, *o*-nitrophenol- β -galactoside, is added to the plates, followed by incubation at 37°C. The incubation is terminated by addition of sodium carbonate when the average absorbance, as determined by visual observation of the yellow product (*o*-nitrophenol), is within a standard range. Absorbance at a wavelength of 415 nm is then quantified spectrophotometrically. β -Gal rates are calculated according to the following equation:

$$\beta\text{-Gal rate} = \frac{\beta\text{-Gal absorbance} \times 10^5}{\beta\text{-Gal incubation time}}.$$

For each set of replicate wells, normalized response is calculated according to the following equation:

$$\text{Normalized response} = \text{LUC units}/\beta\text{-Gal rate}$$

EVALUATION

Agonist activity is determined by examining the amount of LUC expression (normalized response). The effective concentration that produces 50% of the maximal response (EC_{50}) is quantified. The efficacy is a function of the LUC expression relative to the maximal LUC expression produced by the reference agonist, e. g., progesterone. Antagonist activity is determined by testing the amount of LUC expression in

the presence of a fixed concentration (equal to its agonist EC_{50}) of reference agonist. The concentration of test compound that inhibits the gene expression induced by the reference compound by 50% is quantified (IC_{50}). In addition, the efficacy of antagonists is determined as a function of maximal inhibition (LUC expression = basal activity).

CRITICAL ASSESSMENT OF THE METHOD

See Sect. N.2.1.1.2. for the importance of controlling transfection efficacy.

MODIFICATIONS OF THE METHOD

For transactivation studies, hPRA-A-MMTV-LUC and hPR-B-MMTV-LUC and stably co-transfected CHO cells were used by Dijkema et al. (1998) and Schoonen et al. (1998).

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N.3.2.1.3

Alkaline Phosphatase Assay

PURPOSE AND RATIONALE

Progestins induce the *de novo* synthesis of alkaline phosphatase (Di Lorenzo et al. 1991, 1993). The progestin induction of the nonspecific tissue alkaline phosphatase is not altered by other steroid hormones or synthetic analogs. This finding has been used by Sobek et al. (1994), Pathirana et al. (1995), and Li et al. (1997) to measure the progestogenic and antiprogestogenic actions in a microplate assay.

PROCEDURE

T47D cells are grown in 96-well plates to near confluence and then treated with 0.1%–0.2% alcohol (control) or steroid agonists; otherwise R 5020 plus potential antagonist for 2 days. The cells are then fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 min at 15°C, washed with 200 μ l phosphate-buffered saline and stored at –90°C. After thawing, each well is incubated with 100 μ l *p*-nitrophenyl phosphate (1 mg pNPP/ml) in DEAM (1 M diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2$, and 20 μ M $ZnSO_4$). The enzyme reaction is allowed to proceed in the dark at 37°C. Formation of *p*-nitrophenol is monitored periodically at 405 nm in a microplate reader. In each microtiter plate, blanks, *p*-nitrophenol, and alkaline phosphatase standards are measured together with the samples. One unit of alkaline phosphatase is defined as the amount of enzyme capable of transforming 1 μ mol of substrate in 1 min at 37°C. Enzyme assays are performed under conditions of linearity relative to the substrate and to the concentration of proteins.

EVALUATION

Progestin concentrations corresponding to half the maximal increase in alkaline phosphatase activity (ED_{50}) are calculated graphically and by computer-assisted analysis using the ImmunoFit EIA/RIA analysis software (Beckman Instruments).

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N.3.2.2

In Vivo Methods

N.3.2.2.1

Clauberg (McPhail) Test in Rabbits

PURPOSE AND RATIONALE

In this historical bioassay, Clauberg (1930a, 1930b, 1930c, 1930d) first described the histological changes of the endometrium in estrogen-pretreated rabbits after administration of progestational compounds. The test was further studied by Butenandt et al. (1934) and systematically examined by McPhail (1934) who introduced scoring the changes of the endometrium.

PROCEDURE

Immature female rabbits (Yellow Silver or New Zealand strain), weighing 550–650 g, receive daily injections of 5.0 μ g estradiol benzoate per animal in sesame oil solution for a period of 6 days (priming). From days 7–12 (treatment for 5 days) the test compound or standard is administered at several doses, three to four animals per group. Standard doses are: 0.02, 0.08 and 2.0 mg progesterone per day and animal s.c. in sesame oil solution, or 0.01, 0.02, and 0.04 mg medroxyprogesterone acetate orally. Controls receive either the vehicles or estradiol benzoate only. At autopsy on day 15, both horns of the uterus are removed and fixed in 10% formalin. Sections from the middle part of each uterine horn are low examined by histology.

EVALUATION

Pretreatment with oestradiol stimulates uterine weight developmental (priming), progestagens induce further proliferation and secretory transformation. The following scores are established:

- 0 ramification of the uterus mucosa, but no proliferation (estrogen treatment only)
- 1 slight proliferation of the uterus mucosa
- 2 medium proliferation of the uterus mucosa, slight additional ramification
- 3 pronounced proliferation of the uterus mucosa
- 4 very pronounced proliferation of the uterus mucosa, pronounced ramification.

The scores from each dosage group are averaged. Dose–response curves are constructed in order to calculate potency ratios versus the standard (progesterone). The morphological evaluation needs to be done by an experienced investigator. Preliminary experiments with standard drugs and photographic documentation of the histological findings are recommended.

Similar studies were reported by: Zondek 1935; Junkmann 1957; Elton and Edgren 1958; Miyake 1962; Wiechert and Neumann 1965

MODIFICATIONS OF THE METHOD

In order to study the prolonged activity of progestogen esters, the animals are primed with estradiol for 6 days, followed by a single subcutaneous injection of the test compound or hydroxyprogesterone caproate standard. Daily treatment with estradiol is continued and the rabbits sacrificed at increasing intervals up to 4 weeks.

McGinty et al. (1939) established a local **progestional test** which involves the direct injection of progesterone into a uterine segment. The test is performed in immature rabbits primed for 6 days with estrogen. On day 7, the uterus is exposed by laparotomy. The upper middle segment of each horn is ligated without disturbance of blood circulation. A solution of the gestagen in oil is injected into the lumen of one segment through the lower ligature, which is closed tightly after the injection. In the opposite horn, only the vehicle is injected. The animals are sacrificed 3 days later and sections of the horn are evaluated histologically according to the McPhail scores (Tayama et al. 1979).

Pincus et al. (1957) improved the quantitative aspects of the McPhail test with the planimetric measurement of the endometrial proliferation.

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N.3.2.2.2

Endometrial Carbonic Anhydrase Assay

PURPOSE AND RATIONALE

Carbonic anhydrase activity in the endometrium is increased after administration of progesterone. Carbonic anhydrase activity in the endometrium of rabbits was used for a quantitative progestin assay (Lutwak-Mann 1955; Pincus et al. 1957; Elton and Edgren 1958; Miyake and Pincus 1958; Eisler 1964).

PROCEDURE

Immature female rabbits are estrogen primed and progestin treated as described in the Clauberg test. After sacrifice of the animals, the uteri are opened longitudinally. Endometrium is dissected, weighed and homogenized with a tenfold volume in a glass homogenizer. After centrifugation, carbonic anhydrase is determined in the supernatant with the colorimetric method of Philpot and Philpot (1936) using bromothymol as indicator.

EVALUATION

Mean values of carbonic anhydrase activity/g wet tissue are calculated and potency ratios for test compound and standard established.

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N.3.2.2.3

Deciduoma Formation

PURPOSE AND RATIONALE

This is a classical bioassay for progestagens. The deciduoma tests are based on the fact that the endometrium of the estrogen-primed, progesterone-treated rodent is sensitive to local stimuli such as scratching (Astwood 1939), chemical irritation, and electrical stimulation. The effect of local irritation produces a deciduoma, similar to the formation of a maternal placenta (Astwood 1939; Elton and Edgren 1958; Zarrow et al. 1958; Miyake 1962; Eisler 1964; Sreenivasulu 1993).

PROCEDURE

Adult female Sprague-Dawley rats weighing 200–250 g are ovariectomized and 1 week later treated with 0.5 μ g estradiol/animal once daily subcutaneously for 4 days, followed by 9 days of progesterone or the test compound in various doses. The uterus is exposed on day 5 of progesterone treatment and 1.0 mg histamine dihydrochloride is injected into the lumen of one horn. The animals are sacrificed after the last treatment. Both uterine horns are removed and weighed. The degree of deciduoma formation is evaluated by the percentage increase in weight of the histamine-injected uterine horn as compared with the untreated control horn.

EVALUATION

Dose–response curves are established plotting percentage increase in weight of the treated uterus horn versus logarithm of dose of test compound and standard in order to calculate potency ratios.

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N.3.2.2.4**Pregnancy Maintenance Assay****PURPOSE AND RATIONALE**

This is a historical bioassay for progestational activity. In the rat, ovariectomy performed during the first half of pregnancy terminates gestation, but ovariectomy during the second half of pregnancy does not result in abortion, because the placenta now provides estrogens and progesterone for maintenance of pregnancy. In early pregnancy, maintenance of pregnancy after ovariectomy can be achieved by sufficient exogenous progestin with and without estrogen (Stucki 1958; Miyake 1962; Eisler 1964; Hebborn 1971; Phillips et al. 1984; Kuhnz und Beier 1994).

PROCEDURE

Mature Sprague-Dawley female rats are inseminated by being placed with males overnight. Presence of sperm is assessed by vaginal lavage and, if positive, is considered to be day 1 of pregnancy. On day 8, the females are ovariectomized if found pregnant upon examination of the uterus. Test compounds are administered once daily, subcutaneously, for 13 days beginning immediately after ovariectomy. Estradiol (0.1 μ g/day) is administered concomitantly with the test compound. At autopsy on day 21, the presence or absence of implantation sites, and the numbers of live embryos are recorded.

EVALUATION

Normal pregnant rats have an average of 11 implantation sites and about 10 live embryos. Maintenance of pregnancy is assessed with reference to these normal values.

MODIFICATIONS OF THE METHOD

Similar tests have been performed in rabbits (Elton and Edgren 1958), in mice (McGinty 1959), and in hamsters (Shipley 1965). In rabbits, abortion can be induced by intravenous injection of oxytocin from day 30 of pregnancy onwards. The abortifacient effect can be suppressed by progestagens.

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N.3.2.3**Anti-Progestational Activity****N.3.2.3.1****Progesterone Antagonism (Anti-Progestins)****PURPOSE AND RATIONALE**

The anti-progestational activity of a test compound may be determined by antagonism against the effect of progesterone in the Clauberg/McPhail assay in rabbits (N.3.2.2.1) or the McGinty test (N.3.2.2.1) and in the deciduoma formation assay in rats (N.3.2.2.3) (Miyake 1965; Tamaya et al. 1979; Oettel and Kurischko 1980; Philibert et al. 1985; Cook 1994; Teutsch and Philibert 1994).

MODIFICATIONS OF THE METHOD

Progesterone control of cervical ripening in the guinea pig and the tree shrew *Tupaia belangeri* has been used for the evaluation of progesterone antagonists (Chwalisz et al. 1991; Chwalisz 1994). The effects of progesterone antagonists on surgically induced endometriosis in rats have been studied by Stöckemann and Chwalisz (1993).

Michna et al. (1991) developed a bioassay which allows quantification of the antiproliferative potency of progesterone antagonists on the mammary gland in rats. Female Wistar rats with a body weight of 100 g were ovariectomized. One week after ovariectomy the rats were substituted with 10 µg estrone and 3 mg progesterone for 3 days. The animals in the experimental groups simultaneously received the progesterone antagonist. The animals were then sacrificed and the inguinal mammary glands dissected: the right gland for biochemical analysis (DNA), the left for morphometrical analysis. The entire inguinal mammary gland was prepared for conventional paraffin sections and stained with ferric ammonium sulfate. Microphotographs were taken in transmitted light. The number of tubulo-alveolar buds was counted in the whole mount preparations using a 40-fold magnification. In the neighborhood of the inguinal node, a square of 2.5 mm² was quantified and calculated for a tissue volume of 100 mm³ in more than ten animals. The antiproliferative action of progesterone antagonists on the amount of tubulo-alveolar buds was estimated for an 80% confidence interval of mean inhibition.

CRITICAL ASSESSMENT OF THE METHOD

This method is very detailed and time-consuming, it appears to be suitable for the advanced stages of drug evaluation for anti-progestins. In contrast to the only biochemical assessment, the method provides detailed information which is also applicable to toxicological evaluations.

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N.3.2.3.2**Luteolytic Activity of Prostaglandins****PURPOSE AND RATIONALE**

Synthetic prostaglandins are used for synchronization of estrus and for treatment of anestrus caused by a persistent corpus luteum in cattle. Furthermore, synthetic prostaglandins were studied for termination of early pregnancy in humans (Karim et al. 1977; Takagi et al. 1977, 1978; Topozada et al. 1979, Dwivedy 1979). The most useful animal model for luteolytic activity is the hamster (Gutknecht et al. 1971; Labhsetwar 1971, 1972a; Dukes et al. 1974; Bartmann et al. 1979; Galliani et al. 1984; Roy et al. 1987). The effects of prostaglandins on reproductive function in other species such as rat (Fuchs et al. 1974), mouse (Labhsetwar 1972b), and guinea pig (Blatchley and Donovan 1969) have also been studied.

PROCEDURE

Adult female golden hamsters (*Mesocricetus auratus*), weighing approximately 100 g, with regular, 4-day estrus cycles are housed under controlled light and temperature conditions and given a standard diet. They are caged with fertile males on the day before expected vaginal discharge and the next morning vaginal smears are taken. If clumps of spermatozoa are found in the smear, this day is designated as day 1 of pregnancy (see similar procedure in rats). Groups of

ten animals are treated on days 4, 5, and 6 with four different doses of standard (prostaglandin $F_{2\alpha}$) or test compound. On day 13 of pregnancy, the hamsters are sacrificed and the number of implantation scars in the uterus is counted as the parameter of luteolytic activity causing early termination of pregnancy.

EVALUATION

The luteolytic activity is expressed as the median effective dose for termination of pregnancy in 50% of the treated animals (ED_{50}).

MODIFICATIONS OF THE METHOD

The mode of action of luteolysis by prostaglandins has been studied by various authors both *in vitro* (Speroff and Ramwell 1970; O'Grady et al. 1972; Henderson and McNatty 1975; Kenny and Robinson 1986; Brambaifa 1988) and *in vivo* (Pharriss and Wyngarden 1969; Johnston and Hunter 1970; McCracken et al. 1970; Chatterjee 1973; Buhr et al. 1983; Torjesen and Aakvaag 1984, 1986).

Cao and Chan (1993) investigated the effects of oxytocin and luteal prostaglandins on the functional regression of the corpus luteum in pseudopregnant rats.

Motta et al. (1996) studied the effect of an oxytocin receptor antagonist on ovarian and uterine synthesis and release of prostaglandin $F_{2\alpha}$ in pseudopregnant rats.

Stocco and Deis (1998) examined the participation of intraluteal progesterone and prostaglandin $F_{2\alpha}$ in LH-induced luteolysis in pregnant rats.

Luteolysis by prostaglandins in the rhesus monkey has been studied by Auletta and Kelm (1994) and Auletta et al. (1995).

The ewe as a model for regulation of luteal regression has been recommended by Hoyer (1998).

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N.4

Testicular Steroid Hormones

N.4.0.1

Castration of Male Rats (Orchiectomy)

PROCEDURE

Castration of young male rats is performed with minimal bleeding in animals weighing less than 60 g. The animal is anesthetized. A small transversal incision is made in the skin on the ventral site over the symphysis. The testis lying in the scrotum is gently pushed into the abdominal cavity. With a pair of fine forceps, the abdominal wall is opened. The epididymal fat pad, easily seen, is grasped with the forceps and the testis with the epididymis is pulled out from the wound. The ductus deferens with the testicular vessels is crushed with artery forceps and the testis together with the epididymal fat pad cut off with a pair of fine scissors. There is almost no bleeding in young animals. In older animals, ligation of the testicular vessels together with the ductus deferens may be necessary. The same procedure is performed on the other side. The skin wound is closed with one or two wound clips. The animal recovers immediately. With some skill, the operation can be performed very rapidly (Bomskov 1939).

MODIFICATIONS OF THE METHODS

Dorfman (1969) recommended removing the testes through an incision in the tip of the scrotum. In our hands, the procedure described above was preferable.

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N.4.0.2

Caponizing of Cockerels (Orchiectomy)

PROCEDURE

This is a classical bioassay for androgens. White Leghorn cockerels are used for surgery at approximately 6 weeks of age. The animals, fasted 24 h prior to surgery, were anesthetized with ether and placed on their sides. An incision is made between the last two ribs, the muscle layer is divided, and the incision is pulled apart with small retractors. The testis is found close to the midline of the posterior abdominal wall, alongside the vena cava. The capsule enclosing the testis is cut and the gonad is removed. It is imperative to remove the testis intact, as fragments left behind are usually vascularized and persist, giving rise to incompletely caponized animals. The incision is closed by a suture. The contralateral testis is removed in a similar fashion (Bomskov 1939).

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N.4.1

Androgenic and Anabolic Activity

N.4.1.1

In Vitro Methods

N.4.1.1.1

Androgen Receptor Binding

PURPOSE AND RATIONALE

Rat ventral prostate (Bonne and Raynaud 1974; Liao et al. 1974; Grover and Odell 1975; Ojasoo and Raynaud 1978; Raynaud et al. 1979; Winnecker et al. 1989; Duc et al. 1995) or mouse kidney (Isomaa et al. 1982) serve as sources for androgen receptors. Human androgen receptors have been prepared from trans-

fect COS-1 cells (Teutsch et al. 1994). Labeled androstano-1-one, 5 α -dihydrotestosterone, testosterone, and, more recently, methyltrienolone (R 1881) have been used as radioligands.

PROCEDURE

Androgen Receptor Assay

Cytosol is prepared from ventral prostate glands of adult male rats castrated approximately 24 h before use. The tissue is homogenized in TMDG buffer (10 mM Tris, 20 mM sodium molybdate, 2 mM dithiothreitol, 10% glycerol, pH=7.4) at room temperature using a motor-driven glass homogenizer and centrifuged at 135,000 g for 1 h. Aliquots of the supernatant (cytosol) are diluted to contain 40 mg tissue/ml and incubated for 1 h or overnight with [$^{17}\alpha$ -methyl- 3 H]R 1881 (methyltrienolone, 5 nM final concentration, 87 Ci/mmol, New England Nuclear) in either the absence or presence of increasing concentrations (1 nM to 10 μ M) of R 1881 or test compounds. Because R 1881 binds weakly to progesterone and glucocorticoid receptors, cytosols are pretreated with 1 μ M triamcinolone acetonide to block these interactions. After a 1- or 18-h incubation period, a suspension of dextran-coated charcoal (1% charcoal, 0.05% dextran T-70, 0.05% BSA) is added to the ligand-cytosol mixture and incubated for 5 min. The charcoal is removed by centrifugation at 1500 g for 10 min and the supernatant (protein-bound [3 H]R 1881) counted using 10 ml of scintillation fluid (New England Nuclear) in a liquid scintillation spectrometer.

Nuclear Androgen Receptor Exchange Assay

Ventral prostates are homogenized at 100 mg/ml in hexylene glycol buffer (1 M hexylene glycol, 1 mM MgCl₂, 2.0 mM dithiothreitol, 5.0 mM EGTA, 1.0 mM PIPES, pH=7.4) using a motor-driven ground glass homogenizer. Homogenates are centrifuged at 1500 g for 10 min. The nuclear pellet is washed 3 times in homogenization buffer by gently resuspending the pellet in a Dounce homogenizer and centrifugation at 1500 g for 10 min. The washed nuclear pellet is resuspended in pyridoxal-5'-phosphate extraction buffer (20 mM sodium barbital, 5 mM pyridoxal-5'-phosphate, 5.0 mM dithiothreitol, 1.5 mM EDTA, 150 mM KCl, 20% glycerol, pH=7.4) for 60 min at a final concentration of 60 mg tissue/ml. The extracted nuclei are centrifuged at 25,000 g for 30 min with the resulting supernatant being used in the same single saturating dose assay as described for prostate cytosol.

EVALUATION

Binding of test substances to the androgen receptor (receptor affinity) is quantified by calculating relative binding affinity (ratio of the molar concentration of unlabeled R 1881 to test substance required to inhibit the binding of [3 H]R 1881 by 50% after correction for nonspecific binding) and equilibrium inhibitory binding constant ($K_i = IC_{50}/(1 + C)/K_d$, where C = the concentration of [3 H]R 1881 and the K_d for R 1881 is 1.3 nM).

In the nuclear androgen receptor exchange assay treatment group means are compared to control means, using ANOVA and Dunnett's multiple comparison tests.

MODIFICATIONS OF THE METHOD

Cell assays and animal assays are described for evaluation of androgens and anti-androgens (Raynaud et al. 1975; Liang et al. 1977; Sivelles et al. 1982; Liao et al. 1984; Traish et al. 1986; Stobaugh and Blickenstaff 1990; Christiansen et al. 1990; Humm and Schneider 1990; Neubauer et al. 1993).

Brown et al. (1981) studied anti-androgen effects on androgen receptor binding in cultured human newborn foreskin fibroblasts.

Tezón et al. (1982) studied the intracellular distribution of the androgen receptor in the rat epididymis under the influence of androgens and anti-androgens.

The use of tritiated 7 α ,17 α -dimethyl-19-nortestosterone for the assay of androgen receptors was recommended by Schilling and Liao (1984).

Characterization and expression of a cDNA encoding the human androgen receptor was described by Tilley et al. (1989).

Hoyle et al. (1993) recommended 7 α -methyl-17 α -(E-2'-[125 I]iodovinyl)-19-nortestosterone as radioligand for the detection of the androgen receptor.

Structure-affinity relationships of various steroids structurally related to nomegestrol and progesterone for [3 H]testosterone binding to rat ventral prostate cytosol were reported by Botella et al. (1987).

Molecular cloning of human androgen receptor complementary cDNA has been reported by Chang et al. (1988) and Lubahn et al. (1988).

DNA-binding of androgen receptor overexpressed in COS-1 cells has been reported by Von Krempelhuber et al. (1994).

Toth et al. (1995) studied *in vitro* binding of 16-methylated C-18 and C-19 steroid derivatives to the androgen receptor using cytosol of castrated rat prostate and [3 H]R 1881 as radioligand.

Chang et al. (1995) reviewed the structure and function of the androgen receptor and its role in the function of the mammalian system.

The interaction of androgen receptors with the androgen-response element in intact cells was investigated by Karvonen et al. (1997).

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N.4.1.1.2

Transactivation Assay for Androgens

PURPOSE AND RATIONALE

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone-responsive elements of hormone-regulated genes, in-

ducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows determination of the agonistic and also the antagonistic potency of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

PROCEDURE

Vector Construct

CV-1 cells are stably transfected with the rat androgen receptor and pMMTV-CAT7. The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

The culture medium of CV-1 cells stably transfected with the rat androgen receptor and pMMTV-CAT7 is supplemented with 400 µg/ml G418 (Gibco BRL) and 5 µg/ml puromycin.

Stable and transient transfections are performed using Lipofectin reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80% confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 µg hAR expression plasmid and 5 µg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 µg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish, gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10% fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3% charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. Cells are cultured with 1% ethanol

as a negative control for the reporter gene induction. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37°C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

EVALUATION

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to demonstrate the potency of the test hormone. The synthetic androgen R 1881 (10^{-10} to 10^{-6} mol/l) is used as the standard.

For anti-androgenic activity, CAT activity in the presence of 0.5 nmol/l R 1881 is set as 100% and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the antihormone.

CRITICAL ASSESSMENT OF THE METHOD

See N.2.1.1.2.

MODIFICATIONS OF THE METHOD

Warriar et al. (1993) examined the ability of dihydrotestosterone (DHT) and various antiandrogens to stimulate or to inhibit the transcription activation of mouse mammary tumor virus-bacterial chloramphenicol acetyltransferase (MMTV-CAT) in CV-1 cells.

White et al. (1994) described a simple and sensitive high-throughput assay which can be adapted for several classes of steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein-Barr virus episomal vector which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses high level of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone

(DHT). These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase (CAT) assay.

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N.4.1.2

In Vivo Methods

N.4.1.2.1

Chicken Comb Method for Androgen Activity

PURPOSE AND RATIONALE

This is a historical bioassay based on the response of the comb of castrate cockerels (capons). The method has been used by many authors to assay androgenic activity and has been found to be extremely useful for the isolation and structural elucidation of natural androgens. Many modifications have been published (reviewed by Dorfman 1969).

PROCEDURE

Prior to assay, the surface area (sum of the length plus height of each individual comb) is determined by a millimeter rule placed directly on the comb. The capons are injected q.d. intramuscularly for 5 days consecutively with a solution or suspension of the test compound and reference animals are treated with the androgen standard in 1 ml olive oil. Then 24 h after the last injection, the combs are measured again and the growth of the comb is expressed as the sum of length and height in millimeters. Groups of eight animals are used for at least two doses of the test compound and the standard. In this method, animals can be used repeatedly.

EVALUATION

The experimental parameter is the surface area of the comb. The mean values of each group are calculated, dose–response curve for the test compound and the standard are plotted, and potency ratios are calculated where possible.

Similar studies were reported by: Gallagher and Koch 1935; Greenwood et al. 1935; Oesting and Webster 1938

MODIFICATIONS OF THE METHOD

The hormones, dissolved in oil, have been applied locally to the capon's comb instead of by injection. A greater sensitivity has been achieved with this modification (Fussgänger 1934; McCullagh and Cuyler 1939).

Newly hatched chicks of either sex have been used to study the growth of combs after systemic or local administration (Frank et al. 1942; Dorfman 1948). White Leghorn chicks are used at an age of 2–3 days. They are kept in a brooder with thermostatic control. An oily solution (0.05 ml) of the test compound or the standard is applied to the comb daily for a period of 7 days. Then 24 h after the last application, the animals are autopsied. Body weights are determined. The combs are removed by two longitudinal incisions along the base of the comb at its juncture with the scalp. The comb is freed from the scalp, touched lightly on a towel to remove blood and weighed. Dose–response curves are established.

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N.4.1.2.2

Weight of Androgen-Dependent Organs in Rats

PURPOSE AND RATIONALE

Androgens affect the development of secondary sex organs in the male rat (ventral prostate, seminal vesicles). The growth of the ventral prostate, the seminal vesicles and the musculus levator ani is dependent on the presence of male sexual hormones. Weight development of the musculus levator ani was considered to indicate anabolic activity, and weight development of the ventral prostate and seminal vesicles was considered to indicate androgenic activity (Dorfmann 1969).

PROCEDURE

Immature male Sprague-Dawley rats weighing about 55 g are orchietomized. Animal body weight is recorded at the beginning and at the end of the experiment.

The animals are treated with test compounds by gavage (orally) in 0.5 ml 0.5% carboxymethylcellulose or by subcutaneous injection in 0.2 ml sesame oil suspension once per day over a period of 10 days. Testosterone is given in doses of 0.02, 0.1, and 0.5 mg per animal subcutaneously, and methyltestosterone orally in doses of 0.25, 1.5, and 5 mg per animal. Controls receive the respective vehicle. Ten animals are used for each group. On day 10 at autopsy, the seminal vesicles, ventral prostate, and levator ani muscle are dissected out and weighed. The seminal vesicles are squeezed and dried to remove the fluid.

Dissection of the levator ani muscle is performed after removal of the skin in the scrotal area between the base of the penis and the anus. The posterior aspect of the perineal complex is cleared of fat and connec-

tive tissue with forceps, particular care being taken to expose the constrictions at either end of the levator ani where it joins the bulbocavernosus muscle. The rectum is transected just caudad to the point where the musculus levator ani loops around it dorsally. The body of the levator ani is then freed of the rectum and is removed by incisions at the points of attachment to the bulbocavernosus muscle. The levator ani is cleared of any connective tissue and weighed to the nearest 0.1 mg.

EVALUATION

The ratio of organ weight to body weight is calculated for every animal and each organ (relative organ weights). Dose–response curves are constructed for each organ comparing the test compound with the standard in order to calculate potency ratios. An increase in the weight of seminal vesicles and ventral prostate indicates androgenic activity, whereas an increase in the weight of musculus levator ani is considered to indicate anabolic activity.

In evaluating steroids for possible use as anabolic agents, Hershberger et al. (1953) suggested the use of the levator ani:ventral prostate ratio, which is defined as the ratio of the increase in levator ani weight divided by the increase in ventral prostate weight.

The method has been used by several authors: Korenchevsky and Dennison 1935; Eisenberg and Gordon 1950; Junkmann 1957; Kincl 1965; Eisler 1964; Dorfmann 1969; Kuhnz and Beier 1994.

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N.4.1.2.3**Nitrogen Retention****PURPOSE AND RATIONALE**

Anabolic agents induce positive nitrogen balance in the living organism (Polish 1964; Dorfmann 1969). Many modifications of this assay principle have evolved. Stafford et al. (1954) suggested a method involving the measurement of nitrogen excretion in the castrated rat fed a liquid diet and in nitrogen balance.

PROCEDURE

Twenty-five-day-old rats are castrated and kept untreated for 67 days, reaching about 300 g in body weight on normal laboratory diet. After 67 days, they are changed to a liquid diet, forced-feeding regime. The diet contains carbohydrates and fat, as well as casein and brewer's yeast as a nitrogen source. At the start, the rats receive 10 ml of feed per day, and this is increased to 26 ml per day. Feeding is continued for 30 days with simultaneous administration of the test drug once a day. Twenty-four-hour urine specimens are collected 3 times weekly and analyzed for total nitrogen.

EVALUATION

Indices are calculated, such as greatest daily retention, which is defined as the difference between the lowest daily nitrogen value after the beginning of treatment and the preinjection mean; the total nitrogen retention, which is the sum of the differences between the preinjection excretion and the daily values during the retention period; and the number of days in the retention period.

MODIFICATIONS OF THE METHOD

A method for the assay of anabolic steroids in the monkey (*Macaca mulatta*) has been suggested by Stucki et al. (1960). Nitrogen retention expressed as total nitrogen retained per day during the treatment period is chosen as the end point.

CRITICAL ASSESSMENT OF THE METHOD

The assay is time-consuming and labor-intensive, and its use is no longer recommended.

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N.4.2**Anti-Androgenic Activity****N.4.2.0.1****General Considerations**

Anti-androgens may exhibit their activity both peripherally on androgen-dependent tissues and by feedback action at a central site (Neumann et al. 1970; Mainwaring 1977; Neri 1977; Neumann et al. 1977; Raynaud et al. 1977; Neumann 1985; Moguilewski and Bouton 1988). They compete with the peripheral androgen receptors and thus inhibit the effect of endogenous or exogenous androgens. Centrally, they inhibit gonadotropin secretion and thereby diminish testosterone production by the gonads. In addition to their effects on reproduction and accessory sexual organs, anti-androgens inhibit sebum production (anti-acne drugs) and delay androgen-dependent hair loss (alopecia). The methods to detect and quantify gonadotropin inhibition are described in Sect. N.7.1.8.

Inhibition of 5 α -reductase, an enzyme located in tissues such as the prostate, is one pharmacological approach to inhibit benign prostate hyperplasia in men. Such inhibitors reduced the conversion from testosterone to 5 α -dihydrotestosterone (DHT).

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N.4.2.1

In Vitro Methods

N.4.2.1.1

Inhibition of 5 α -Reductase

PURPOSE AND RATIONALE

Testosterone is converted to 5 α -dihydrotestosterone (DHT) by the enzyme 5 α -reductase which is specifically localized in some androgen-responsive target tissues (e. g., prostate, seminal vesicle, epididymis, skin and sebaceous glands), whereas in other androgen-sensitive tissues, such as the skeletal muscles and the central structures, the androgenic hormone is testosterone. Inhibition of 5 α -reductase provides a selective approach to androgen deprivation in DHT-target tissues, such as the prostate. The 5 α -reductase inhibitors are applied in the therapy of benign prostate hyperplasia.

PROCEDURE

5 α -Reductase preparations are obtainable from prostates of various species, such as human, dog, and rat.

Frozen human prostates from benign prostatic hyperplasia patients are thawed and minced with a pair of scissors. The minced tissue is homogenized in three tissue volumes of medium A (20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose, 1 mM dithiothreitol and 50 μ M NADPH) with a Brinkmann Polytron and a glass-glass homogenizer. The homogenate is centrifuged at 140,000 *g* for 60 min and the pellets are washed with approximately three tissue volumes of medium A. The washed pellets are suspended at a concentration of 5–10 mg protein/ml in 20 mM potassium phosphate, pH 6.5, containing 20% glycerol and 1 mM dithiothreitol.

Dog prostatic particulates are prepared from either fresh or frozen specimens of male mature dogs as described for human prostate. The washed pellets are suspended in medium A at a concentration of 30–60 mg protein/ml.

Ventral prostates from male Sprague-Dawley rats weighing 400 g are processed as described for human prostate, except that medium A without NADPH is used throughout the procedures. NADPH prevents inactivation of human and dog 5 α -reductases during the preparation, the rat enzyme however is stable without the coenzyme.

For the 5 α -reductase assay, reaction solutions are prepared in duplicate tubes containing 1 μ M [¹⁴C]testosterone, 1 mM dithiothreitol, 40 mM buffer (potassium phosphate, pH 6.5, for the rat and for the dog enzymes; Tris-citrate, pH 5.0, for the human enzyme), prostatic particulate (1 mg protein), and NADPH (50 μ M for reaction with rat enzyme, 500 μ M for reaction with human and dog enzyme) in a final volume of 0.5 ml. Test compounds or standard as inhibitors are added in 5 μ l ethanol at concentrations between 10⁻⁹ and 10⁻⁵ M. The control tubes receive the same volume of ethanol. The reactions for the rat and dog enzymes are started by the addition of the prostatic particulates. The human prostatic particulate is premixed with NADPH before starting the reaction. The reactions are linear for at least 1 h at 37°C. The reactions are carried out for 10–30 min and are stopped with 2 ml ethyl acetate containing testosterone, 5 α -dihydrotestosterone, and androstenedione (10 μ g each). After centrifugation at 1000 *g* for 5 min, the ethyl acetate phase is transferred to a tube and evaporated under nitrogen to dryness. The steroids are taken up in 50 μ l ethyl acetate. The solutions are applied to Whatman LK5DF silica plates and the plates are developed in either ethyl acetate:cyclohexane (1:1) at 25°C or chloroform:methanol (96:4) at 4°C. The plates are air-dried and the chromatography is repeated. Nonradioactive steroid standards are located by UV and by spraying with 1% CeSO₄/10% H₂SO₄ solution followed by heating. The radioactivity profiles are determined by scanning the plates or by scraping the silica in sections and counting in a scintillation counter. 5 α -Dihydrotestosterone is the only radioactive product for the rat and human enzymes. With the dog enzyme 5 α -dihydrotestosterone, 3 α ,17 β -androstenediol, androstan-3,17-dione, and androstenedione are formed. The radioactivities of the first three products are combined for the calculation of the 5 α -reductase activity.

EVALUATION

IC₅₀ values are calculated based on at least five dilutions of test preparations or standard.

MODIFICATIONS OF THE METHOD

The method has been used by several authors: Bruchovsky and Wilson 1968; Corvol et al. 1975; Brooks et al. 1981; Liang et al. 1985; Rhodes et al. 1993; di Salle et al. 1993; Sudduth and Koronkowski 1993; di Salle et al. 1994

Using human genital skin fibroblasts and simian COS cells, specific inhibition of 5α -reductase type I has been observed (Hirsch et al. 1993).

Wennbo et al. (1997) reported that **transgenic mice** overexpressing the prolactin gene develop dramatic enlargement of the prostate gland.

Sigimura et al. (1994) described age-related changes of the prostate gland in the senescence-accelerated mouse and recommended this strain as a model of age-related changes in the prostate gland.

Neubauer et al. (1993) measured prostatic 5α -reductase in rats both *in vitro* and *ex vivo* and determined *in vivo* uptake of [3 H]testosterone by the prostate.

At least two isoforms of 5α -reductase have been isolated (Andersson and Russell 1990; Jenkins et al. 1992). Recombinant human prostatic 5α -reductase type I and II were expressed using the baculovirus-directed insect cell expression system (Iehlè et al. 1993).

Iehlè et al. (1995) and di Salle et al. (1998) tested synthetic 5α -reductase inhibitors against both isoforms.

Tolman et al. (1995) identified a 4-azasteroid as a scalp isoenzyme selective inhibitor.

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N.4.2.2

In Vivo Methods

N.4.2.2.1

Chick Comb Method

PURPOSE AND RATIONALE

This method is now obsolete for the evaluation of anti-androgens. Several modifications of the chick comb method have been described for anti-androgens applied either systemically or locally to the comb of intact cockerels (Dorfmann 1960, 1969).

PROCEDURE

Male or female, 1- to 3-day-old White Leghorn chicks are housed at constant temperature in a heated incubator. Testosterone is incorporated into the finely ground chick starting mash at a concentration of 80 mg/kg of food to stimulate comb growth. The chicks are placed on this diet on day 1. The anti-androgen to be tested is

dissolved in sesame oil and injected for several days. At 24 hours after the last injection, the animals are sacrificed, the combs removed and blotted to remove blood, and weighed rapidly to the nearest 0.5 mg. Body weights are also determined.

EVALUATION

The results may be expressed as absolute comb weights or as milligram of comb per gram of body weight. Dose–response curves for groups treated with increasing doses of anti-androgen are plotted.

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N.4.2.2.2

Antagonism of Androgen Action in Castrated Rats

PURPOSE AND RATIONALE

In this modification, anti-androgens are administered to reduce or inhibit testosterone action's on androgen-dependent organs.

PROCEDURE

Male Sprague-Dawley rats weighing 50–70 g are orchietomized. Starting 1 day after castration, the rats are injected once daily for 7 days with 0.15 mg testosterone propionate in 0.1 ml sesame oil (agonist action). The test compounds (anti-androgens) are dissolved in sesame oil or suspended and injected subcutaneously daily at a separate site for the same test period of 7 days. Six to ten animals are used per group. At autopsy on day 8, weights of ventral prostate, seminal vesicles and musculus levator ani as well as body weight are recorded.

EVALUATION

The organ weight to body weight ratio is calculated for each order, preferably based on relative organ weight to 100 g of body weight. The inhibition by the anti-androgen is compared with agonist action in the groups of animals receiving testosterone propionate alone. Dose–response curves may be plotted for each organ, and expressed as percentage inhibition of the agonist effect of testosterone by the anti-androgen.

MODIFICATIONS OF THE METHOD

Dorfman (1962) described an anti-androgen assay using the castrated mouse. Weights of prostate and semi-

nal vesicles were determined after injection of the anti-androgen test compounds and simultaneous injections of 2 mg testosterone over a period of 7 days.

APPLICATIONS OF THE METHOD

The intrinsic anti-androgenic activity is an important parameter in the evaluation of the pharmacological activity of H₂-receptor antagonists (Winters et al. 1979; Broulik 1980; Baba et al. 1981; Sivelle et al. 1982; Foldesy et al. 1985; Takeda et al. 1982; Neubauer et al. 1990). In general pharmacology studies, there is rarely any need for specific anti-hormonal tests. However, in toxicology studies, tests for anti-androgen activity may be warranted when the effects on the testes and androgen-dependent organs are found in repeated-dose studies.

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Similar results are found using intact immature female rats.

EVALUATION

Dose–response curves were established for increasing doses of the anti-androgen at a given dose of testosterone propionate or for increasing doses of testosterone propionate at a given dose of the anti-androgen, cyproterone.

CRITICAL ASSESSMENT OF THE METHOD

The method is time-consuming and was directed to the specific investigation of cyproterone and cyproterone acetate in precocious puberty and androgen-dependent disorders.

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N.4.2.2.3

Anti-Androgenic Activity in Female Rats

PURPOSE AND RATIONALE

This is another historical bioassay of interest due to the design of exploring anti-androgenic activity. Neumann and Elger (1966) described a method for testing the anti-androgenic activity of compounds in immature female rats. The inhibition by the anti-androgen cyproterone of the trophic effect of testosterone on uterine and preputial growth was studied in intact as well as in castrated female rats (Neri et al. 1972; Snyder et al. 1989).

PROCEDURE

Female Sprague-Dawley rats weighing 40–45 g are ovariectomized. One week later, the treatment is started for 12 days with daily subcutaneous injections of 0.3 mg testosterone propionate and several doses of the antagonist. Controls receive testosterone propionate only. At autopsy on day 13, the uteri and preputial glands are dissected out and weighed. The weight increase of female accessory sexual organs caused by testosterone's action is dose-dependently reduced by the anti-androgen (in this case, cyproterone).

N.4.2.2.4

Intra-Uterine Feminizing/Virilizing Effect

PURPOSE AND RATIONALE

From clinical observations as well as from experimental data (Neumann and Junkmann 1963; Neumann and Kramer 1964), it is well known that the external genitalia of female fetuses can be masculinized by tumors secreting endogenous androgens or by steroids with androgenic activity. This effect can be antagonized by an anti-androgen.

The method is of interest for assessment of reproductive toxicology.

PROCEDURE

Adult female Sprague-Dawley rats are mated and the beginning of pregnancy is determined by vaginal smears. From day 16 to day 19 of pregnancy, the antiandrogens are administered in various doses subcutaneously in sesame oil. Testosterone propionate is used in doses between 1.0 and 10.0 mg as the androgenic stimulus. The dams are sacrificed on the 20th day of pregnancy and the external genitalia of the female embryos examined. The sex of the embryos is recognized by the presence of ovaries and uterus. A dose of 10 mg

testosterone propionate leads to total masculinization of female embryos with loss of female and the appearance of male sex characteristics. The anogenital distance in female rat fetuses measured macroscopically and microscopically is dose-dependently increased by testosterone propionate. This characteristic androgen effect is diminished by an anti-androgen.

EVALUATION

The androgen-dependent decrease of the anogenital distance in female fetuses by various doses of the anti-androgen is expressed as percentage inhibition of the testosterone-induced virilization.

MODIFICATIONS OF THE METHOD

Feminization of male rats was induced by treatment of pregnant rats during the second half of gestation and of the newborn fetuses during weeks 1–3 post partum with an anti-androgen, e. g., cyproterone acetate (Neumann and Elger 1966; Nishino et al. 1988; review by Neumann 1994). A decrease of the anogenital distance in the male fetuses of anti-androgen-treated rats is expressed as percentage inhibition relative to fetuses from untreated mothers.

In feminized male rats, nipples and associated glandular tissues develop after birth as in normal female rats (Neumann and Elger 1967).

Feminization of male rats treated *in utero* was also observed with nonsteroidal anti-androgens and a 5α -reductase inhibitor (Imperato-McGinley et al. 1992).

REFERENCES AND FURTHER READING

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N.4.2.2.5

Anti-Androgenic Activity on Sebaceous Glands

PURPOSE AND RATIONALE

Bioassays for topical anti-androgens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965), and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. This effect is used for morphometric evaluation of topical anti-androgens.

The method is described in detail in Sect. P.9.1.

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N.4.2.2.6

Anti-Androgenic Activity in the Hamster Flank Organ

PURPOSE AND RATIONALE

This is another bioassay preferably for topical anti-androgens. The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species, these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemic or topical anti-androgens.

The method is described in detail in Sect. P.9.5.

N.4.2.2.7**Effect of 5 α -Reductase Inhibitors on Plasma and Tissue Steroid Levels****PURPOSE AND RATIONALE**

5 α -Reductase inhibitors change the ratio of plasma testosterone to dihydrotestosterone (DHT) as well as the tissue concentrations particularly in the prostate tissue.

PROCEDURE**Treatment of Animals**

Male Sprague-Dawley rats are treated subcutaneously with the 5 α -reductase inhibitor or vehicle beginning on postnatal day 3 until the age of 4 or 7 weeks. After sacrifice, blood is withdrawn for testosterone and DHT determinations (George et al. 1989). Moreover, intraprostatic concentrations of testosterone and DHT are determined as an index of antiproliferative activity (di Salle et al. 1993).

Radioimmunoassay for Testosterone and Dihydrotestosterone

Serum testosterone and DHT are measured by radioimmunoassay (RIA) in serum or serum extracts using specific antisera without prior chromatography (Falvo and Nalbandov 1974). Serum samples of 0.5 ml may be extracted with 2 ml of freshly purified, peroxide-free diethyl ether by shaking for 60 s on a Vortex mixer. The aqueous phase is frozen at -70°C , then the ether phase containing steroids is transferred to conical test tubes, and evaporated under a stream of dry nitrogen. The dry residue is redissolved in BSA/phosphate buffer (1% BSA = bovine serum albumin) for RIA. [1,2,6,7- ^3H]-Testosterone or [1,2,6,7- ^3H]-dihydrotestosterone and specific antisera are added and tubes incubated over a period of 24 h at $+4^{\circ}\text{C}$ under nonequilibrium conditions. Bound hormone and free hormone are separated by adsorption on dextran-coated charcoal. The activity of each sample is determined by beta-spectrometry.

Commercially available RIA kits can be used with suitable validation.

EVALUATION

The hormone concentrations in the sample are calculated from a standard curve by a computer program (e.g., RIA-Calc, LKB), using appropriate control sera. The ratios of testosterone to DHT in rats treated with different doses of 5 α -reductase inhibitors are compared with those of vehicle-treated intact control rats.

MODIFICATIONS OF THE METHOD

di Salle et al. (1998) measured prostatic concentrations of testosterone and 5 α -dihydrotestosterone in rats by specific RIAs after treatment with a dual type I and II 5 α -reductase inhibitor. Similar measurements of tissue testosterone:DHT ratios have been performed in dogs, in the context of pituitary down-regulation of androgen secretion by luteinizing-hormone-releasing hormone (LHRH) agonists.

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N.5**Thyroid Hormones****N.5.0.1****General Considerations**

The purpose of this section is twofold: to describe the methods for determination of hormones secreted by the thyroid gland, and to describe investigation of thyroid function in experimental models. For information on the hypothalamic–pituitary–thyroid system and its investigation, see the sections on anterior pituitary hormones (N.7) and hypothalamic hormones (N.9).

The thyroid gland secretes two types of hormones: the **thyroid hormones**, i.e., **l-thyroxine** (T_4), and **triiodothyronine** (T_3) which have metabolic functions and are involved in neuronal development, and the **calcitropic hormone, calcitonin**. The functional system for metabolic regulation subserved by the thyroid hormones is entirely different from the complex system for regulation of calcium and phosphate balance fulfilled by (thyo)calcitonin, parathormone from the parathyroid glands, and the calciferol hormones (formerly vitamin D) produced by the liver and kidneys and activated in the skin.

The main biological effects of T_3 and T_4 are on growth and development (e.g., maturation of tadpoles), their calorogenic effect (increase of basic metabolic rate), cardiovascular function (increased

sensitivity of the heart to catecholamines), and metabolic functions (lipid, carbohydrate, and collagen metabolism). The primary feedback effect is inhibition of thyroid-stimulating hormone (TSH) secretion. These effects can be used to test thyroid hormone analogs and metabolites. The thyroid hormones regulate iodine uptake and utilization in the thyroid, and their action can be inhibited by antithyroid drugs.

Historical bioassays rely on morphogenesis and neuronal development in **amphibia** (Biedl 1916; Pitt-Rivers and Tata 1959; Copp et al. 1962; Turner and Premachandra 1962). Thyroid hormones induce premature metamorphosis in amphibian tadpoles. Since the first observation by Gudernatsch (1913a, 1913b) this phenomenon has been studied by numerous workers with the purpose of adapting this response for the assay of thyroidal substances (Bomskov 1937). Within a short period of time, the treatment with thyroid hormones induces the transformation of the **tadpole** into a small frog with growth of limbs, lungs, and other terrestrial accoutrements, and stimulates the synthesis of enzymes mediating morphogenesis and transformation.

The **axolotl** (*Ambystoma mexicanum* or *tigrinum*) has been used as a test object to study metamorphosis induced by thyroid hormones. This animal loses the gills and forms lungs, changing the shape of its tail at the same time (Huxley and Hogben 1922; Zavadovsky and Zavadovsky 1926; Haffner 1927).

Another principle action of T_3 and T_4 is metabolic activation and increased energy expenditure. Kreitmair (1928) standardized thyroid preparations using the **weight loss of guinea pigs** after 1 week of treatment as a parameter. A guinea pig unit was defined as the dose which reduces the body weight of guinea pigs with an initial weight of 250–300 g within 7 days by at least 10%.

A different functional role is subserved by calcitonin. The **hypocalcemic hormone calcitonin** was discovered by Copp (Copp et al. 1962; Copp 1964). Calcitonin originates from parafollicular C-cells of the thyroid. Its functional antagonist is parathyroid hormone. The bioassay of calcitonin preparations is performed by assessing their ability to **lower the plasma calcium** in the rat. Assay of serum (thyro)calcitonin has a significant diagnostic role for thyroid carcinoma.

As with other hormones, research methods have progressed from bioassays of thyroid hormone action to direct measurement of the thyroid hormones (thyroxine and triiodothyronine) and their metabolites, to studies on enzymatic steps in thyroid hormone synthe-

sis and inactivation, to the identification of thyroid hormone receptors as members of the super family of nuclear receptors, and to signaling induced by binding of thyroid hormones to their receptors.

Thyroid Hormone Receptors

Nuclear triiodothyronine binding proteins were purified and characterized by Torresanai and Anselmet (1978). Ichikawa and DeGroot (1987a, 1987b) described the purification and characterization of rat liver nuclear thyroid hormone receptors and thyroid hormone receptors in a human hepatoma cell line. Apriletti et al. (1988) reported large-scale purification of the nuclear thyroid hormone receptor from rat liver and sequence-specific binding of the receptor to DNA. Ichikawa et al. (1988) and Ichikawa and Hashizume (1991) published methods of an aqueous two-phase (dextran and polyethylene glycol) partitioning study of nuclear thyroid hormone receptors. Glucocorticoids, other steroid hormones, thyroid hormones and vitamin-derived hormones (including retinoids) all exert their effects by the regulation of hormone-responsive target genes within the cell nucleus. William and Franklyn (1994) reviewed the physiology of the steroid–thyroid hormone nuclear receptor superfamily. A nuclear hormone receptor-associated protein that inhibits transactivation by the thyroid hormone and retinoic acid receptors was described by Burris et al. (1995). Two different genes encode two different thyroid hormone receptors, thyroid hormone receptor- α and thyroid hormone receptor- β and these two thyroid hormone receptors are often co-expressed at different levels in different tissues. Chiellini et al. (1998) designed a high-affinity subtype-selective agonist ligand for the thyroid hormone receptor- β . The expression of thyroid hormone receptor isoforms in rat growth plate cartilage *in vivo* was described by Ballock et al. (1999). Yuan et al. (1998) described a component of a thyroid hormone receptor-associated protein (TRAP) co-activator complex which interacts directly with nuclear receptors in a ligand-dependent fashion. The sequence of the thyroid-hormone-response element and the recruitment of retinoid X receptors for thyroid hormone responsiveness was investigated by Wu et al. (2001).

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N.5.0.2

Thyroidectomy

PURPOSE AND RATIONALE

Experiments for pharmacological evaluation of thyroid hormones and analogs were performed in thyroidectomized rats. Bomskov (1937) described the method of thyroidectomy in various animal species, such as tadpoles, frogs, birds, goats, dogs, cats, rabbits, guinea pigs, rats and mice, based on the clinical experience with thyroid resection in humans.

PROCEDURE

The thyroid in rats consists of three lobes (left, median, and right). The rat is anesthetized, e. g., with pentobarbital, and placed on a surgical table. The fur of the neck is removed with electric clippers and the area disinfected. A median skin incision of 2.0 cm length is made. On both sides large salivary glands and maxillary lymph nodes are found. They are pushed aside, making visible the musculus hyoideus covering the trachea. This muscle is split in the midline. The isthmus of the thyroid connecting both lobes is located below the thyroid cartilage. The lobes and the isthmus are separated with blunt forceps from the trachea and the blood vessels ligated. Alternatively, the thyroid can be removed by electrocauterization. In most cases, the parathyroid glands are severed by the operation, and postoperative substitution with calcium lactate 1% in drinking water is advised.

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N.5.1

In Vivo Tests for Thyroid Hormones

N.5.1.1

Oxygen Consumption

PURPOSE AND RATIONALE

Basal metabolic rate, oxygen consumption and CO₂ production are increased by thyroid hormones. This has been used for diagnostic procedures in humans as well as for evaluation of thyroid hormones and their derivatives in animals (indirect calorimetry). The historical method based on survival time of mice placed individually into tightly closed glass jars (Smith et al. 1947; Basil et al. 1950; Gemmill 1953) was modified, measuring time until occurrence of convulsions. The method was based on the increase in oxygen consumption associated with the markedly increased metabolic rate at high doses of thyroid hormones.

PROCEDURE

This is a description of the now obsolete assay: mice are placed individually into 200-ml wide-necked bottles. The bottom of the bottles is covered with filter paper to soak up the urine. The bottles are tilted to an angle of 60° and rotated five times per minute in a special apparatus. The time until asphyctic seizures occur is noted. Immediately after observation of seizures, the mouse is released for recovery. Due to the defined muscle work, the time to seizures is shortened in controls to 20–30 min.

EVALUATION

Average time to seizures was calculated and dose-response curves were established.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Bomskov 1937; Lilienthal et al. 1949; MacLagan and Sheahan 1950; Reineke and Turner 1950; Anderson 1954; Heming 1964; Turner 1969

Several apparatuses have been designed to measure oxygen consumption in animals, e. g., by Holtkamp et al. (1955).

Stock (1975) described an automatic, **closed-circuit oxygen consumption apparatus** for small animals. A Perspex animal chamber is surrounded by a water jacket except for one end, which has a removable cover plate. This cover, as well as allowing access to the chamber interior, also holds the connections for the oxygen delivery line and the pressure line. For experiments involving injections, infusions, and blood sampling, catheters are passed through, and sealed into rubber bungs which are then forced into holes in the cover plate. A rubber gasket forms an airtight seal between the cover and the chamber. Within the chamber, the animal is supported on a wire grid over a layer of self-indicating soda lime and silica gel. A major determinant of sensitivity in this system is the dead space of the chamber. Chambers with internal dimensions of 20 × 10 × 10 cm are suitable for animals such as mice and rats up to about 250 g body weight. Fixed volumes of oxygen are introduced into the chamber by an automatic syringe dispenser (Fisons Scientific) which draws pure oxygen from a spirometer through a drying tube filled with silica gel. When chamber pressure exceeds atmospheric by about 3 mmH₂O, the microdifferential pressure switch (KDG Instruments) inactivates the dispenser. The dispenser is reactivated when the pressure differential drops below this threshold value. The volume of oxygen dispensed is adjusted to the smallest volume that, with a single action of the syringe, will return chamber pressure to above the threshold value. The particular dispenser used in this system has the advantages of being (1) gas tight and (2) when activated will complete its pump cycle even if the chamber pressure exceeds the threshold value in midcycle. A discrete fixed volume of oxygen is delivered each time it is activated. To obtain the rate of oxygen consumption it is merely necessary to record the pump rate.

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N.5.1.2

Inhibition of Iodine Release

PURPOSE AND RATIONALE

The thyroid gland has a high avidity for iodine, uptake of which may be measured by isotope-labeled iodine (^{131}I), in a dose-related and time-dependent manner. The release of ^{131}I from the thyroid in rats is inhibited by treatment with thyroxine (Wolff 1951), and the degree of inhibition is related to the dose administered (Perry 1951). This phenomenon was used to compare activity of thyroid hormone derivatives with the standard thyroxine.

For analytical and diagnostic purposes, direct quantitation of thyroid hormones is now achieved by methods such as radioimmunoassay and HPLC chromatography, and by measuring feedback inhibition of thyroid hormones directly via the decrease in serum TSH.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g are fed a commercial laboratory chow without or with addition of 0.03% propylthiouracil (reference compound for thyroid peroxidase inhibition). Food is withheld 8 h before the injection of $25\ \mu\text{C}^{131}\text{I}$ or ^{125}I intraperitoneally. Radioactivity over the thyroid region of the neck is determined 40 h later (if necessary under sedation). This reading is taken as time zero and all fur-

ther counts made at 24-h intervals may be expressed as a percentage of time-zero counts after correction for physical decay of the ^{131}I isotope. After the reading at time zero, the diet is changed to a feed containing 0.03% propylthiouracil, and several doses of the test preparation or the standard are injected subcutaneously at 24-h intervals up to a total of four doses. The daily loss of ^{131}I is inversely proportional to the dose of thyroid hormone.

EVALUATION

Percentage of time-zero counts after 96 h of ^{131}I remaining in the thyroid after the last of four doses is plotted against logarithm of dose. From these dose–response curves, potency ratios are calculated.

The method has been used by several authors: Reineke and Turner 1950; Anderson 1954; Turner and Premachandra 1962

CRITICAL ASSESSMENT OF THE METHOD

The assay described here was used for quantitative estimates and has now been replaced by analytical determination of thyroid hormone contents. For human drug formulations, bioequivalence studies are required when generic formulations are assessed. This approach of measuring the uptake and release of labeled iodine may be modified for short-term uptake of ^{131}I or ^{125}I as a parameter of thyroid peroxidase inhibition by antithyroid drugs, and other drugs affecting thyroid function.

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N.5.1.3

Anti-Goitrogenic Activity

PURPOSE AND RATIONALE

Thyroid weight and size are controlled by the action of thyroid-stimulating hormone (TSH) on thyroid tissue. In rats, increased secretion of TSH induces thyroid enlargement and weight increase within a few

days (addressed as goiter formation). In normal animals the secretion of TSH by the pituitary is regulated by feedback of thyroid hormones. The administration of goitrogenic compounds which block thyroid hormone synthesis and/or secretion reduces the concentrations of circulating thyroid hormones (T_4/T_3) and their pituitary effect (negative feedback inhibition of TSH secretion), releasing TSH from its feedback inhibition. The TSH rise induces hyperplasia of the thyroid follicles as indicated by the dose-related increase of thyroid weight. Hyperplasia is prevented by injection of thyroxine, triiodothyronine or thyroid hormone analogs.

PROCEDURE

Male Sprague-Dawley rats weighing 150–180 g are used in groups of eight to ten animals. During the treatment period, 0.1% propylthiouracil (PTU) is added to the food or to the drinking water, in order to achieve a stable baseline of thyroid weight. Over a period of 2 weeks, the rats are treated (preferably by gavage) with various doses of the test compound or the thyroxine standard (10–40 $\mu\text{g}/\text{kg}$). PTU controls are treated with the suspension medium or saline injections only. At autopsy on day 14, the thyroid glands are dissected out and weighed rapidly to avoid evaporation loss. Thyroids may also be lyophilized first to weigh dry matter. The two- to three-fold increase of thyroid weight by PTU is reversed dose-dependently to normal values by thyroid active substances.

EVALUATION

Dose–response curves are plotted and potency ratios with confidence limits may be calculated.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Reineke et al. 1945; Pitt-Rivers and Tata 1959; Turner and Premachandra 1962; Wiberg et al. 1964; Ortiz-Caro et al. 1983; Pisarev et al. 1994

The effect of PTU-induced baseline suppression is monitored and ascertained by measuring serum TSH, T_4 and T_3 . The dose-related inhibition of the TSH rise by thyroid substances is used as the parameter to assess goiter prevention.

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N.5.1.4

Tensile Strength of Connective Tissue in Rats, Modified for Thyroid Hormones

These studies are an example of evaluating the biological effect of high doses of thyroid hormones on tissues other than those involved in the increase of metabolic rate. Thyroid hormone secretion affects almost all tissues in the body, and high doses may exert unwanted effects on connective tissue.

PURPOSE AND RATIONALE

The decrease of tensile strength after a single high-dose injection of thyroid hormones is dose dependent and can be used for evaluation of thyroid hormone derivatives. Short-term treatment with corticosteroids increases the strength of connective tissue (Vogel 1969). This effect is antagonized by thyroid hormones (Ther et al. 1963; Vogel and Ther 1964). Thyroid hormones per se have a biphasic effect: short-term treatment decreases the dose-dependent tensile strength of epiphyseal cartilage, tail tendons and skin strips, whereas treatment over 10 days increases tensile strength, probably mediated by activation of endogenous adrenal secretion (similar to the effect of corticosteroid treatment).

PROCEDURE

Male Sprague-Dawley rats weighing 110 ± 10 g are injected subcutaneously with thyroid hormones (dose range of the standard L-triiodothyronine 0.1–1.0 mg/kg). After 24 h the animals are sacrificed and the tensile strength of distal femoral epiphyseal plates, tail tendons, or skin strips are tested as described in Sects. N.2.1.3.2, N.2.1.3.3, N.2.1.3.4, and P.11.2.1.1.

EVALUATION

Doses–response curves of test compounds and standard are established and potency ratios may be calculated. T_3 (L-triiodothyronine) is about 3 times more active than T_4 (levothyroxine), in accordance with its

metabolic activity. The mechanism of action of this short-term effect of thyroid hormones on connective tissue has not been explored.

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N.5.2

Antithyroid Drugs

N.5.2.1

General Considerations

Antithyroid drugs interfere with synthesis, release, and/or the peripheral action of the thyroid hormone, lowering the basal metabolic rate. They are used in the treatment of thyroid disorders (hyperthyroidism). The suppression of T₄/T₃ secretion reduces thyroidal inhibition of the pituitary gland, increasing TSH secretion, and then induces the goitrogenic response. This response was used to detect antithyroid drugs and has been widely used for screening procedures. It is, however, nonspecific and may be caused by several different mechanisms, including enzyme induction of glucuronyltransferases. The goitrogenic response is of considerable interest in toxicology, because it may be produced by several compounds during early drug evaluation which modify the biosynthesis and/or inactivation of thyroid hormones in an unexpected manner.

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N.5.2.2

Inhibition of Iodine Uptake in Rats

PURPOSE AND RATIONALE

Propylthiouracil (PTU) and a wide spectrum of drugs may inhibit thyroid hormone synthesis. Some of these

drugs are used to treat thyrotoxicosis. As a consequence of thyroid peroxidase inhibition, the iodine uptake by and content in the thyroid is decreased. This phenomenon is dose dependent and may occur at lower doses than those increasing thyroid weight in rats (McGinty and Bywater 1945). The historical parameter of iodine content has been replaced by measuring the uptake and release of ¹³¹I.

PROCEDURE

Groups of male Wistar rats age 26–28 days, weighing 40–45 g, are placed into metabolism cages. They are fed normal diet, and potassium iodide is added to the drinking water. In one modification of the method (for toxicology studies), the test compounds or the reference standard (several concentrations) may be added to the diet over a period of 10 days, and the amount of compound ingested by each rat is then calculated from the total food consumption over 10 days and expressed in milligram daily per kilogram of body weight. After 10 days of treatment, the rats are sacrificed and the thyroids dissected free from adjacent tissue and capsule. The thyroid is weighed and iodine content determined. In daily doses of between 0.1 and 10.0 mg/kg, thiouracil decreases the iodine content of the thyroid in a dose-dependent manner. Definitely higher doses are necessary to increase thyroid weight.

EVALUATION

Dose–response curves of test compounds and reference standard are plotted, and potency ratios with confidence limits may be calculated.

MODIFICATIONS OF THE METHOD

Walker and Levy (1989) used implantable pellets of propylthiouracil to induce thyroid dysfunction in rats. Uptake of labeled iodine is measured instead of iodine content. Release of labeled iodine may be stimulated by protirelin (TRH) injection in order to assess thyroid function, or as a quantitative bioassay for the effect of the hypothalamic hormone TRH.

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N.5.2.3

Antithyroidal Effects in Animal Assays

The oxygen consumption in iodine-treated mice has been used as a bioassay, modified for antithyroid activity.

PURPOSE AND RATIONALE

The historical bioassay is based on oxygen consumption, which is increased in acutely potassium-iodide-treated mice, resulting in a decrease of asphyxiation time (thyroid activation). This effect is dose-dependently antagonized by antithyroidal compounds, and the time to convulsions is prolonged due to the reduced metabolic rate. The methods based on increased oxygen consumption after thyroid hormones (Sect. N.5.1.1) are applied.

MODIFICATIONS OF THE METHOD

Thyroid weight was an early parameter for the detection of antithyroid activity. Rabbits treated with goitrogenic compounds or fed exclusively with cabbage (Chesney et al. 1928; Marine et al. 1929) showed an up to tenfold increase of thyroid weight, histologically manifested as hyperplasia without colloid formation. These phenomena were reversed by iodine treatment (Bomskov 1937).

Goiter formation as a side-effect of non-steroidal anti-inflammatory drugs was studied by Müller et al. (1985).

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N.5.3

Calcitonin

N.5.3.1

General Considerations

The calcitropic hormone (thyro)calcitonin was discovered in C-cells of the thyroid gland by Copp (Copp et al. 1962; Copp 1964, 1994). This hypocalcemic hypophosphatemic principle of the thyroid gland (Austin and Heath 1981) was designated thyrocalcitonin by Hirsch et al. (1964), Munson and Hirsch (1966), Raisz et al. (1967), and MacIntyre (1992). Its calcitropic effects on bone and kidney function are opposite to those of the parathyroid hormone. Calcitonin originates from parafollicular C-cells of the thyroid. Calcitonin secretion can be evaluated *in vitro* using the isolated perfused porcine thyroid (Pento 1985). Radioimmunoassays for calcitonin are available (Tashjian and Voelkel 1979), and species-specific methods for calcitonin determination need to be considered. Assays for calcitonin receptors have been described (Nissenson et al. 1985). Surveys on the effects of exogenous calcitonin was given by Defetos (1989); Braga (1994); Wallach et al. (1999). The biology and clinical relevance of calcitonin gene peptides has been reviewed (Reginster 1993; Silverman 2003; Zaidi et al. 1990).

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N.5.3.2

Decrease of Serum Calcium in Rats

PURPOSE AND RATIONALE

The bioassay of calcitonin preparations is performed using their ability to lower the plasma calcium in the rat. This procedure has also been adopted by pharmacopoeias, using the International Reference Preparation for Calcitonin (porcine) consisting of freeze-dried purified pork calcitonin, and the International Reference Preparation for Calcitonin (salmon) consisting of freeze-dried purified synthetic salmon calcitonin. These assays for calcitonin quantitation, however, have now been replaced by a physicochemical method for pharmaceutical quality control. Either intravenous or subcutaneous administration can be chosen. International standards for salmon calcitonin, eel calcitonin, and the Asu¹⁻⁷ analogue of eel calcitonin have been elaborated (Zanelli et al. 1990). A second international standard for porcine and human calcitonins has been established by an international collaborative study group based on the *in vivo* rat hypocalcemia bioassay (Zanelli et al. 1993).

PROCEDURE

Groups of at least five female Wistar rats, weighing 100–120 g, are used. Three doses of standard preparation (1, 3 and 9 mU per rat) and three doses of test preparation are injected intravenously. Then 1 h after injection, blood is withdrawn under light anesthesia. Plasma calcium is determined by flame photometry or by atomic absorption photometry.

EVALUATION

Dose–response curves of decreases in plasma calcium are established and potency ratios with confidence limits are calculated.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Kumar et al. 1965; Munson et al. 1968; Rittel et al. 1976; Schwartz et al.

1981; Michelangeli et al. 1983; Findlay et al. 1985; Buck and Maxl 1990; Deming et al. 1994

Yates et al. (1990) assessed the acute hypocalcemic responses to single subcutaneous injections of calcitonin preparations in intact young male ICR Swiss mice weighing 12–20 g.

Calcitonin of the stingray and of the goldfish were characterized by Sasayama et al. (1992, 1993).

Kapurniotu and Taylor (1995) performed *in vitro* hypocalcemic assays in mice by analysis of serum calcium 1 h after subcutaneous injection of lactam-bridged analogs of human calcitonin.

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N.5.3.3

Effect of Calcitonin on Osteoclasts in Vitro

PURPOSE AND RATIONALE

Calcitonin acts primarily by inhibition of osteoclastic bone resorption (Friedman and Raisz 1965; Aliapoulos et al. 1966). Zaidi et al. (1990, 1994) reported the development and validation of three microbioassays for calcitonin based on calcitonin-induced inhibition of the activity of isolated osteoclasts.

PROCEDURE

Femora and tibiae are removed from newborn Wistar rats. The bones are freed from adherent soft tissues and cut across their epiphyses in HEPES-buffered Medium 199 supplemented with heat-inactivated fetal calf serum, benzyl penicillin (100 µU/ml) and streptomycin (100 µg/ml). The osteoclasts are mechanically disaggregated by curetting the bones of each rat with a scalpel blade into 1 ml medium and agitating the suspension with a pipette. Larger fragments are allowed to settle for 10 s, before the supernatant is dropped onto appropriate substrate (bone slices, plastic Petri dishes or glass coverslips).

Motility-Based System

The morphological appearance of stained osteoclasts is used as an index to assess the state of cytoplasmic activity. Osteoclasts are settled on coverslips in microtiter wells and are incubated for 20 min at 37°C. The coverslips are removed, washed with Medium 199 and placed in separate wells, each containing 100 µl medium.

Following a further incubation for 30 min (37°C), serial dilutions (tenfold) of salmon or human calcitonin or test preparations or appropriate dilutions of plasma samples are added. The cells are finally incubated for 2 h, fixed in 10% glutaraldehyde and stained with toluidine blue. The state of motility of each osteoclast on each coverslip is scored by observing the characteristic shape change these cells undergo when motility is inhibited; a motile cell is characterized by a smooth outline with increased staining intensity over

all or part of its periphery, whereas a immotile cell typically shows an irregular pale outline without ruffled edges. The number of immotile cells is counted on each coverslip and expressed as a percentage of the total number of cells counted.

Cytoplasmic Spreading System

Osteoclasts are settled in tissue culture dishes (35 mm) and are incubated at 37°C for 20 min to allow sedimentation and attachment. The cells are then washed with Medium 199 and 2 ml of the same medium is placed in each well. The dishes are placed in the incubation chamber of an inverted phase-contrast microscope. Images of the osteoclasts are recorded on a time-lapse video recorder. A tracing of their outlines is transferred through a digitizing system into a computer, programmed to measure the area within each tracing.

The outlines of each osteoclast are recorded before or after the addition of calcitonin or vehicle to the cultures. For each variable, the outline of six osteoclasts is traced after a 60-min incubation in the chamber and again 40 min following the addition of the hormone. The mean surface area covered by six osteoclasts after incubation is expressed as a percentage of the mean surface area of the osteoclasts before the addition of hormone or vehicle.

Bone Resorption System

Specimens of human femoral cortical bone are obtained from donors (patients who died without evidence of bone disease). The adherent soft tissue is removed and the bone cortex cut longitudinally into slices (0.1 mm thick). The slices are then cut into pieces (approximately 3 mm²). They are cleaned by ultrasonication (15 min, in sterile distilled water), dehydrated by immersion in 80% aqueous ethanol for 2 h, and stored to dry at room temperature. Osteoclasts isolated in Medium 199 are dropped onto 12–16 bone slices placed in a well of an 18-mm multi-well dish. Following incubation (37°C, 15 min), slices are removed, and washed gently in Minimal Essential Medium supplemented with 10% FCS and antibiotics as described above. They are placed in separate wells, each well containing five to six slices in 900 µl medium. After further incubation (37°C, 10% humidified CO₂, 10 min), 100 µl of medium containing the test concentration of the hormone or the test solution is added. Human PTH₍₁₋₃₄₎ (0.1 U/ml) is used to assess functional effects of contaminating osteoblasts.

The calcitonin analogs are tested at various concentrations (tenfold dilutions). Finally, bone slices

are incubated overnight (37°C, 10% humidified CO₂, 18 h). The cells are fixed in glutaraldehyde, stained with toluidine blue and examined by transmitted light microscopy. Osteoclasts and mononuclear cells are counted. The slices are then bleached by immersion in sodium hypochlorite solution for 30 min and dehydrated in 80% aqueous ethanol. Finally, they are sputter coated with gold, randomized and examined in a scanning electronic microscope. The numbers of osteoclastic excavations, each defined by a continuous border, are counted. The area of bone surface resorbed is calculated by tracing the outline of the concavities into a digitizing tablet, linked to a microcomputer. Resorption surface areas may be expressed as a percentage of the mean of the control response.

EVALUATION

Data of each assay are analyzed using classical methods for analysis of parallel line assays. Estimates of relative potencies are calculated from parallel log dose–response lines of test preparations and a reference preparation.

Osteoclasts are mechanically disaggregated from neonatal rat long bones and dispersed at low densities on slices of devitalized bovine cartilage bone. The resulting areas of bone excavation are quantified with micrometric precision by scanning electron microscopy together with computer-assisted image analysis. These findings are used to develop a formal bioassay for calcitonin.

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assay (Findlay et al. 1980, 1983, 1985; Grauer et al. 1992; Sexton and Hilton 1992; Blind et al. 1993).

PROCEDURE

The human breast cancer cell line T47D was originally established from a pleural effusion from an infiltrating ductal breast cancer (Horwitz et al. 1978). For binding experiments, cell monolayers are washed with 0.02% EDTA before treatment with 0.125% trypsin in 0.02% EDTA for 2 min at 37°C, addition of complete medium before centrifugation at 200 g, and resuspension in complete medium.

Iodination of calcitonin is performed with ¹²⁵I using the chloramine-T method.

For binding experiments T47D cells suspended in isotonic buffer are added to ¹²⁵I-labeled salmon calcitonin mixed with varying concentrations of unlabeled calcitonin or analogs and incubated at 20°C for 1 h. Nonspecific binding is assessed as the binding of ¹²⁵I-labeled salmon calcitonin in the presence of excess (2 µg/ml) unlabeled salmon calcitonin.

Stimulation of adenylate cyclase in intact T47D cells by calcitonin analogs is assessed by measuring [³H]cAMP production in cells prelabeled with [³H]adenine. Cellular ATP pools are labeled by incubation with 2,8-[³H]adenine (0.5–2 µCi/ml) for 2 h at 37°C in 12-well culture dishes in RPMI 1640 medium containing 0.1% BSA. Cells are then washed twice with serum-free medium and incubated for a further 20 min in medium containing 0.1% BSA and 1 mM isobutylmethylxanthine (IBMX) before treatment with calcitonin and its analogs for 10 min at 37°C in the same buffer. Incubations are terminated by removing medium and adding 100 µl 20% trichloroacetic acid at 4°C. This is followed by 800 µl of a 5 mM solution of ATP, ADP, AMP, cAMP, and adenine. The [³H]cAMP is isolated by chromatography on Dowex and alumina. Radioactivity is counted in a scintillation counter.

EVALUATION

For both parameters full dose–response curves are generated and the concentrations required for half-maximal responses are calculated.

N.5.3.4

Receptor Binding and cAMP Accumulation in Isolated Cells

PURPOSE AND RATIONALE

The human breast cancer cell line T47D responds to calcitonin and its analogs by receptor binding and accumulation of cAMP. This can be used as a biological

MODIFICATIONS OF THE METHOD

Yates et al. (1990) measured stimulation of adenylate cyclase activity by calcitonin analogs in primary cultures of mouse renal cortex which were prepared according to the methods of Fukase et al. (1982) from 4-week-old ICR Swiss mice and used at confluence after 4 days of culture.

A radioreceptor assay for potency determinations of formulations of salmon calcitonin was described by Sjödin et al. (1990).

Albrandt et al. (1993) cloned two receptors with high affinity for salmon calcitonin from the nucleus accumbens region of rat brain.

Likewise, Sexton et al. (1993) identified in rats two isoforms of the calcitonin receptor, designated C1a and C1b.

Functional aspects of the isoforms C1a and C1b were discussed by Martin et al. (1995).

The calcitonin receptor isoforms C1a and C1b were localized in rat brain using *in vitro* autoradiography by Hilton et al. (1995).

Keustner et al. (1994) cloned and characterized a second form of the human calcitonin receptor from T47D cells.

Sexton et al. (1994) assayed the cloned renal porcine calcitonin receptor cDNA expressed by transient transfection in COS-1 cells or stable transfection in HEK-293 cells for interaction with calcitonin, amylin and calcitonin gene-related peptide. The results suggested that amylin may act as a natural ligand for the renal porcine calcitonin receptor.

The various pathways in signal transduction by calcitonin were discussed by Horne et al. (1994).

Houssami et al. (1994) found that different structural requirements exist for calcitonin receptor binding specificity and adenylate cyclase activation.

Suva et al. (1997) synthesized benzophenone-containing calcitonin analogs and tested them for receptor binding and stimulation of cAMP accumulation.

Povzek et al. (1997) investigated the structure–function relationship of salmon calcitonin analogs as agonists, antagonists, or inverse agonists in heterologous calcitonin receptor expression systems using two calcitonin receptor cell clones, B8-H10 and G12-E12, which express about 5 million and 25,000 C1b receptors/cell, respectively.

The location of the phosphorylation of the human calcitonin receptor by multiple kinases was studied by Nygaard et al. (1997).

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N.6 Parathyroid Hormone

N.6.0.1

General Considerations

The primary function of parathyroid hormone (PTH) is to maintain a constant concentration of Ca²⁺ in the extracellular fluid. Processes that are regulated include the absorption of Ca²⁺ from the gastrointestinal tract, the deposition and mobilization of bone Ca²⁺, and the excretion of Ca²⁺ in urine, feces, sweat, and milk. PTH is the functional antagonist of calcitonin. The most prominent effect is to promote the mobilization of Ca²⁺ from bone. In the kidney, tubular reabsorption of Ca²⁺ is increased and tubular reabsorption of phosphate is inhibited. Clinically, idiopathic or post-operative hypoparathyroidism results in hypocalcemia followed by tetany.

PTH is used for treatment of hypoparathyroidism, and the symptoms of the disease can also be effectively antagonized by oral administration of dihydrotachysterol. A survey on the structure and function of the parathyroid gland in animals was published by Capen and Rosol (1989). The biologically active synthetic human parathyroid hormone 1–34 fragment (active sequence of PTH) is used for diagnostic testing (Mallette 1988).

An experimental model for secondary hyperparathyroidism with elevated levels of parathyroid hormone in rats has been described by Sancho et al. (1989).

Radioligand assays for parathyroid hormone receptors have been described (Habener and Potts 1976; Nissenson et al. 1985; Schneider et al. 1993).

Immunoassays for PTH are those measuring intact hormone (N-terminal, intact) and those measuring inactive fragments and partial sequences of the intact hormone (mid-region, C-terminal, polyvalent) (Endres et al. 1989). An immunochemiluminometric assay has been described by Klee et al. (1992).

Parathyroid hormone-related protein (PTHrP) was first identified and cloned from malignant tumor cells and tissues from patients with a syndrome called humoral hypercalcemia of malignancy (Mose-

ley et al. 1987; Strewler et al. 1987; Suva et al. 1987). PTHrP-(1–34) and PTH-(1–34) act via a single species of cloned receptor (Abou-Samra et al. 1992; Schipani et al. 1993), although other studies have shown that specific receptors for each of these peptides exist (Usdin et al. 1995; Behar et al. 1996; Bergwitz et al. 1997; Yamamoto et al. 1997; Fukayama et al. 1998). PTHrP is an example of the widening array of sequences identified by molecular endocrinology, with functional characterization of their endocrine role often following after a considerable delay.

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- 1997; Yamamoto et al. 1997; Fukayama et al. 1998; Yaghoobian and Drueke 1998).
- Behar et al. (1996b) reported ligand binding in stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor, and HEK/BP16 cells that express the hPTH₂ receptor.
- The PTH₂ receptor has about 50% amino acid sequence identity with the PTH₁ receptor and is not activated by PTHrP, but by a peptide isolated from bovine hypothalamus, TIP39, which is only distantly related to PTH and PTHrP (Usdin 2000).
- The PTH₃ receptor has been isolated from zebrafish (Rubin and Juppner 1999).
- It is part of the problem of advanced research that cloning of receptors frequently precedes the validation of physiological relevance of receptor subclasses.

N.6.0.2

Receptor Binding Assay for PTH

PURPOSE AND RATIONALE

In many instances, the presence of receptors is explored as the counterpart of the presence of hormonally active substances in tissues and organs. However, the presence of binding sites does not immediately indicate functional activation of the respective tissue, and there are indeed circulating “free receptors” which have been identified as subunits of fragments of “whole receptors” located in cell membranes. Cloned receptors are frequently used for advanced research, to identify compounds which might interact with functional receptors in tissues, and acquire relevance as pharmacological tools for example for high-throughput screening. Furthermore, using radioligand assays to determine the receptor affinity of PTH analogs may provide helpful structure–activity information. Three receptors that are activated by PTH have been cloned. PTH and PTHrP bind to the PTH₁ receptor, for which many studies are published (Abou-Samra et al. 1992; Uneno et al. 1992; Schipani et al. 1993; Ureña et al. 1993; Kaufmann et al. 1994; Schermer et al. 1994; Orloff et al. 1995; Usdin et al. 1995; Bergwitz et al. 1996; Gardella et al. 1996; Yasuka et al. 1996; Bergwitz et al. 1997; Bisello et al. 1997; Guo et al.

PROCEDURE

Stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor (Pines et al. 1994), and HEK/BP16 cells that express the hPTH₂ receptor (Behar et al. 1996a) are maintained in DMEM supplemented with 10% FBS.

The cells are incubated with [¹³¹I]PTH-(1–34) (100,000 cpm/well) with or without competing unlabeled PTH-(1–34) or other ligands in binding buffer for 2 h at room temperature. Cells are washed twice with PBS, then solubilized in 0.5 ml of 0.1 M NaOH. Aliquots are taken for determination of bound radioactivity by γ -counting.

EVALUATION

Specific binding is expressed as counts per minute bound per well (raw data) and converted to the percentage specific binding of radioligand. Affinity constants and binding capacity of these receptors in tissue may be calculated, and dose–response curves of ligands are compared as a first step towards proof of signaling and biological activity.

MODIFICATIONS OF THE METHOD

McCuiag et al. (1994) reported the molecular cloning of the gene encoding the receptor for the parathyroid hormone/parathyroid hormone-related peptide in the mouse.

Inomata et al. (1995) characterized a PTH receptor with specificity for the carboxy-terminal region of PTH-(1–84).

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N.6.0.3

PTH Assay by Serum Calcium Increase

PURPOSE AND RATIONALE

The classical method by Collip and Clark (1925) involves measurement of the rise in serum calcium after administration of parathyroid extracts to dogs. Hamilton and Schwartz (1932) use rabbits treated with oral loads of calcium chloride. The intact rat is very insensitive to injected parathyroid hormone; however, parathyroidectomy produces an increase in sensitivity (Holtz 1937; Davies and Gordon 1953; Davies et al. 1954; Thorp 1969; Zull and Malbon 1976).

PROCEDURE

Male Wistar rats weighing 200–250 g are anesthetized with pentobarbital sodium intraperitoneally. Parathy-

roidectomy is performed by cauterization. After a recovery period of 1 week, blood is withdrawn by retro-orbital puncture (baseline). Various doses of the test preparation or standard are injected subcutaneously to groups of six to ten animals. Blood samples are obtained again 21 h later. Serum calcium is determined by flame photometry. The increase of calcium 21 h after PTH injection is calculated for each animal.

EVALUATION

Mean values of the increase in serum calcium are plotted versus logarithm of dose, and potency ratios versus PTH standard may be calculated for the test compounds.

MODIFICATIONS OF THE METHOD

An increase of whole-body calcium and skeletal mass after repeated-dose treatment with parathyroid hormone in normal rats and in rats with osteoporosis induced by pregnancy and lactation under a low-calcium diet was found by Hefti et al. (1981).

Parathyroid hormone prevented bone loss and augmented bone formation in ovariectomized rats (Kalu et al. 1990; Liu and Kalu 1990).

The active sequence of PTH has been used instead of the full secreted sequence.

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N.6.0.4

Serum Phosphate Decrease After PTH

PURPOSE AND RATIONALE

Tepperman et al. (1947) developed a method using the fall in serum inorganic phosphorus in the rat after injection of parathyroid hormone (Thorp 1962).

PROCEDURE

Male Wistar rats weighing 150–200 g are fed Purina dog chow for at least 2 weeks prior to the experiment. During the experiment only water is allowed. Blood samples (baseline and PTH stimulated, 3 h after PTH) are taken from the tail and 0.6 ml is collected from each rat into tubes, centrifuged for 10 min and 0.2-ml samples of the serum are pipetted into 6 ml of 10% trichloroacetic acid; solutions are centrifuged and 5-ml aliquots of the protein-free solution are used for the estimation of inorganic phosphorus, by the method of Fiske and Subbarow (1925) for example. Serum phosphorus is measured before and 3 h after subcutaneous administration of various doses of test preparation or standard.

EVALUATION

Dose–response curves showing a linear relationship between log dose and response are suitable for the calculation of potency ratios.

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N.6.0.5

cAMP Release in Isolated Perfused Rat Femur

PURPOSE AND RATIONALE

This is an *in vitro* assay. The effect of parathyroid hormone and analogs on release of cAMP from adult bone can be measured in a perfusion system of isolated rat femora (Sugimoto et al. 1985; Lopez-Hilker et al. 1992).

PROCEDURE

Five-week-old Wistar rats anesthetized with pentobarbital (45 mg/kg) are heparinized and used as donors, the femora are removed. Adhering muscles are stripped from the bone. A hole with about half of the depth of the cortex is made with a fine drill at the nutrient foramen below the femoral neck. Then a 21-gauge needle is inserted into this hole and fixed by dental cement to avoid leakage of the perfusate. The bone is then placed in an apparatus for liver perfusion and perfused at a flow rate of 1 ml/5 min by a pump with Krebs-Ringer-bicarbonate, continuously gassed with 95% O₂ and 5% CO₂ and containing 1 mg/ml glucose. Once the perfused bone is assembled, the bone is allowed to equilibrate for 45 min. Samples are collected into a chilled tube for the last 5 min for determination of basal cAMP levels. Then various doses of the test preparations or the standard are infused for 5 min and serial samples are collected every 5 min. In the perfusate, cAMP is measured by radioimmunoassay.

EVALUATION

Time-response curves and dose-response curves are established allowing the calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Nissenson et al. (1981) measured the activation of canine renal cortical plasma membrane adenylate cyclase activity produced by parathyroid hormone standard and test sera from parathyroid venous effluent of patients with primary hyperparathyroidism, in the presence of the hydrolysis-resistant GTP analog 5'-guanylimidodiphosphate.

Gundberg et al. (1995) compared the effects of parathyroid hormone and parathyroid-hormone-related protein on osteocalcin release in the isolated rat hindlimb and in intact and thyro-parathyroidectomized rats.

Saito et al. (1987) established a new biological assay system for simultaneous measurement of bone resorption and bone mineralization in **organ cultures of chick embryonic femur**. Eleven-day-old chick embryonic femur was labeled with ⁴⁵Ca *in vitro*. *T*_{1/2} of calcium efflux was calculated from the sequential release of the label into the medium. Parathyroid hormone increased calcium mobilization, indicating enhanced bone resorption, whereas hydrocortisone and sodium fluoride inhibited bone resorption. Calcitonin was ineffective.

Barling et al. (1989) measured the adenylate cyclase response to parathyroid hormone in cultured rabbit marrow fibroblast cells.

Docherty and Heath (1989) used osteosarcoma cells for an *in vitro* bioassay determining cAMP formation.

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N.6.0.6**Renal and Metatarsal Cytochemical Bioassay****PURPOSE AND RATIONALE**

Cytochemical bioassays using renal and metatarsal tissue are sensitive enough to detect plasma levels of parathyroid hormone and useful for determining the agonist and antagonist activities of fragments and analogs (Chambers et al. 1978; Goltzman et al. 1980; Bradbeer et al. 1988; Loveridge et al. 1991; Zaman et al. 1991; Bourdeau et al. 1990; Wood 1992).

PROCEDURE

For the **renal cytochemical assay**, kidney segments from vitamin-D-depleted guinea pigs are maintained in non-proliferative organ culture for 5 h using Trowell's T8 medium (GIBCO). The medium is then changed for 8 min before exposure to various doses of the parathyroid hormone standard, the parathyroid hormone fragment or the analog for an additional 8 min.

In the experiments for antagonistic activities, each segment is exposed to a single concentration of hPTH-(1–84) (106 fmol/l) or to hPTH-(1–34) (255 fmol/l) in the presence or absence of the antagonist to be tested. The segments are then shock-frozen to -70°C in *N*-hexane before being sectioned at $16\ \mu\text{m}$ on a cryostat. The sections are examined for glucose-6-phosphate dehydrogenase activity using specific staining. The precipitated formazan is quantified in the cells of the distal convoluted tubules by means of a microdensitometer (wavelength 585 nm). Ten readings with each of two duplicate sections are made and the results presented as the mean integrated absorbance $\times 100$ (\pm SEM).

For the **metatarsal cytochemical assay**, the metatarsals of young female Wistar rats weighing 50–100 g are removed and the growth plates are isolated. Only the four longest metatarsals have growth plates large enough to be of use. The metatarsals are maintained individually in nonproliferative organ culture in 5–10 ml Trowell's T8 medium buffered to pH 7.6 in an atmosphere of 95% O_2 , 5% CO_2 at 37°C for 5 h. After the culture period, the medium is removed and each metatarsal exposed to fresh medium (buffered to pH 7.6 by bubbling with a 95% O_2 , 5% CO_2 mixture) containing a low priming dose of PTH (0.5 fg/ml) for 8 min, followed by exposure to known concentrations of a standard PTH preparation or various concentrations of the analog or to dilutions of plasma for 8 min. The metatarsals are then briefly dipped in a 5% solution of polyvinyl alcohol and chilled immediately in *N*-hexane to -70°C .

Each bone is sectioned at $10\ \mu\text{m}$ in a cryostat. The sections are reacted for glucose-6-phosphate dehydrogenase activity in a similar way as described for the renal cytochemical assay. The activity in hypertrophic chondrocytes is linear with respect to time from 10 min, at which time there is enough formazan to be measured, up to 40 min when the density of the formazan formed is too great for a reliable linear response. The enzyme activity in each section is measured in ten individual hypertrophic chondrocytes or osteoblasts lining the metaphyseal trabeculae by scanning and integrating microdensitometry at a wavelength of 585 nm. The results are presented as the mean integrated extinction $\times 100$ (\pm SE) of ten measurements from each of two sections of each metatarsal.

EVALUATION

Dose–response curves are tested for linearity and parallelism and potency ratios are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The cytochemical assays are sensitive but very time-consuming when compared with other analytical methods for the detection of PTH and PTH fragments.

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N.6.0.7

cAMP Accumulation in Cultured Cells

PURPOSE AND RATIONALE

Parathyroid hormone and parathyroid hormone-related protein (PTHrP) stimulate intracellular cAMP accumulation in various cell types in a dose-dependent manner; for example, in PTHrP-overexpressing ROS cells, an osteoblast-like cell line Motomura et al. (1996); in UMR106 cells, a rat osteosarcoma cell line (Oldenburg et al. 1996); in the human osteosarcoma SaOS cell line (Rodan et al. 1987; Fukayama and Tashjian 1994); in COS-7 cells transfected with the human PTH-1 (PTH/PTHrP) receptor and the human PTH-2 receptor (Bergwitz et al. 1997); or in stably transfected human embryonic kidney cells (HEK-293/C-21 cells that express the hPTH/PTRrP receptor and HEK/BP16 cells that express the hPTH2 receptor) (Behar et al. 1996b).

PROCEDURE

Stably transfected human embryonic kidney cells, HEK-293/C-21 cells that express the hPTH/PTRrP receptor (Pines et al. 1994) and HEK/BP16 cells that

express the hPTH2 receptor (Behar et al. 1996a) are maintained in DMEM supplemented with 10%FBS. For cAMP determination, C-21 or BP-16 cells are incubated in 24-well tissue plates with various peptides for 10 min in DMEM in the presence of 1 mM 3-isobutyl-1-methylxanthine. The incubation is terminated by removal of the cell culture medium and addition of perchloric acid (final concentration 30%, vol/vol). Samples are neutralized with potassium bicarbonate and acetylated with acetic anhydride. Total cAMP values (medium plus cells) are determined by RIA. Data are presented as the percentage stimulation, from the ratio of maximal accumulated cAMP levels obtained in the presence of the highest concentration of the agonist PTH-(1–34) compared to the basal cAMP level. The accumulated cAMP levels (picomoles) are calculated per 100,000 cells. Cell number is determined in a Coulter counter.

EVALUATION

Data are presented as the triplicate mean of $([cAMP]_c/[cAMP]_{PTH_{max}}) \times 100 (\pm SEM)$ or sample stimulation vs. maximal PTH induced stimulation. $[cAMP]_c$ is the concentration of cAMP accumulated in response to a given concentration of a ligand, and $[cAMP]_{PTH_{max}}$ is the concentration of cAMP accumulated at the maximal dose (10^{-6} M) of PTH-(1–34).

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N.6.0.8

Bone Anabolic Activity in Ovariectomized, Osteopenic Rats

PURPOSE AND RATIONALE

This is an *in vivo* bioassay which may be used for the beneficial effect of PTH on bone. Vickery et al. (1996) tested an analog of human parathyroid hormone-related protein (1–43) (hPTHrP) in ovariectomized, osteopenic rats.

PROCEDURE

Groups of 3-month-old virgin female rats are subjected to bilateral ovariectomy or sham surgery. The ovariectomized animals are treated by daily subcutaneous injections of 0, 9, 29 or 80 $\mu\text{g}/\text{kg}$ of test compound or human parathyroid hormone-related protein (1–43), starting on day 17 after surgery and continuing for 21 days. On day 12 and 19 following the first day of treatment, animals are dosed intraperitoneally with 6 mg/kg of a 3.75 mg/ml solution of calcein (Sigma) in 2% sodium bicarbonate saline. Body weights are recorded, following an 18-h fast, on the last day of treatment. The animals are sacrificed immediately following the last injection and the femurs, tibiae, and L2 vertebrae are excised.

The distal part of the right femur is cut in half longitudinally after removal of the epiphysis. The bone marrow is flushed out using a stream of water. The trabecular and cortical bone are separated using a dental drill. Calcium is extracted by immersion of the trabecular bone for 3 days and the cortical bone for 5 days in 5% trichloroacetic acid. The calcium content of the extracts is determined. Measurement are expressed as mean \pm SEM in units of milligrams of Ca^{2+} /distal half femur per 100 g of body weight.

For histomorphometry, the left tibiae from sham, ovariectomized and high-dose-treated rats are removed and fixed in formalin. The tibiae are cut with a diamond disc into three pieces: the proximal 1 cm, 2 cm of the diaphysis, and the distal end. The proximal and diaphyseal portions are transferred to Villanueva stain for 72 h (Villanueva and Lundin 1989). The proximal specimen is then dehydrated through increasing con-

centrations of ethanol (70%–100%), defatted in acetone, and embedded in modified methyl methacrylate. Pairs of 5- μm -thick frontal sections are prepared from the anterior aspect with a microtome. The first section is stained with a modified Masson trichrome technique (Goldner 1938) and the second left unstained.

The diaphyseal portion is also dehydrated, defatted and embedded in methyl methacrylate. Five 150- μm -thick sections are prepared, ground to 100 μm thickness, and mounted unstained.

Growth cartilage thickness is measured by finding the distance between the proximal border of the epiphyseal growth cartilage and the metaphyseal junction at 12 places spaced 0.2 mm apart.

On each slide, a rectangular window is defined as the field for evaluation. The top line is drawn under the primary spongiosa, and the second line is drawn parallel to it and 3.5 mm distally. The side lines are drawn near to, but not touching, the endocortical surface.

Histomorphometric variables are calculated for mean trabecular volume and structure (trabecular thickness, number, spacing, mineralizing surface, individual osteoblast activity, and osteoclast surface). Surface-based, bone-volume-based, and total tissue-volume-based bone formation rates are also calculated.

In the cortical bone of the tibial diaphysis, the periosteal and endocortical surfaces are measured separately. Using transmitted light at 25 \times magnification, the periosteal and endo-osteal perimeters are measured. The periosteal single-labeled and double-labeled surface and endo-osteal single-labeled and double-labeled surface are measured using 160 \times magnification.

Vertebral processing and electron microscopy are performed in sham, ovariectomized and compound-treated groups. The second lumbar vertebra is removed, dissected from soft tissue, and split sagittally with a diamond saw. The two halves are fixed in a mixture of glutaraldehyde and formaldehyde for 24–48 h at 4°C. The tissue is decalcified in 10% EDTA in 0.1 M cacodylate, pH 7.2, postfixed for 1.5 h in OsO_4 , stained in block with uranyl acetate, dehydrated and fixed in Epon. Then 50-nm sections are cut and examined in an electron microscope. Microphotographs are printed at a final magnification of 1300 \times . Each microphotograph is overlaid with a grid with a total test line length of $2.22 \times 10^3 \mu\text{m}$. Each intercept of the line with the trabecular surface is scored into one of four categories, depending on the cell type adjacent to the intercept: osteoblasts, osteoclasts, lining cells, no cells. The proportion of the trabecular bone surface area covered by each cell type is estimated by pooling the pictures from

each rat and summing the grid points that intersect each cell type, then dividing by the total number of intersection, excluding the “no cell” counts.

EVALUATION

The treatment groups are compared by one-way analysis of variance (ANOVA) followed by Fisher's least-significance difference test to compare each treatment group with the ovariectomized vehicle control group. For the histomorphometric determinations, the Kruskal-Wallis test is applied to assess *r* group differences. For relative surface area cell coverage estimation, the treatments are compared overall as to the proportions of osteoblasts, osteoclasts, lining cells, and no cells using one-way ANOVA.

MODIFICATIONS OF THE METHOD

Anderson et al. (1990) proposed the ovariectomized, lactating Sprague-Dawley rat as an experimental model for the rapid development of osteopenia which may be used to test the effectiveness of bone-retentive drugs, potentially useful for treating osteoporotic women. Rats were ovariectomized on day 2 postpartum and were kept on a low-calcium diet. Measurements of serum total calcium, ionic calcium, albumin and parathyroid hormone were conducted between days 4 and 21 of lactation.

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N.7 Anterior Pituitary Hormones

Much of the early research on anterior pituitary hormones was performed with extracts obtained from animal pituitary glands, and with animal models for the specific analytical determination of the concentrations of anterior pituitary hormones in serum, in pituitary tissue, and in some cases by measurement of

urinary excretion, for hormones which are excreted during pregnancy, for example. Biosynthetic hormone preparations are now available for human therapy, and species-specific hormone standards are also available for numerous animal species. For an understanding of the development of endocrine research, and the approach to understanding physiology that was necessary, helpful and effective, reference is made extensively to historical methods that are no longer required. As with other fields, the receptors for hypothalamic hormones and anterior pituitary hormones have been characterized and studied, for both a better understanding of regulation, and in several cases structure–activity studies. This approach has also been very helpful in understanding how the sensitivity of the pituitary gland is regulated by both negative feedback of gonadal steroids and the impact of hypothalamic hormones.

N.7.0.1

Hypophysectomy in Rats

PURPOSE AND RATIONALE

This is a classical technique for assessment of the biological effects of pituitary hormones (Collip et al. 1933b; Burn et al. 1952; Vogel 1965; Vogel 1969b). The pituitary gland is removed entirely (anterior and posterior pituitary), the functional deficiencies are identified, and hormone preparations are injected for substitution. Due to the presence of multiple deficiencies after the operation, results by this technique are limited. The approach is of considerable historical interest but has now been replaced by more specific investigation of single hormonal systems. Various techniques of hypophysectomy have been described by Biedl (1916), Thompson (1932), Collip et al. (1933b), Anselmino and Pecharz (1935), and Bomskov (1939) in several animal species, including dog, cat, rabbit, ferret, guinea pig, rat, mouse, chicken, and frog. For some time, hypophysectomy in the rat has been applied to biological standardization of anterior pituitary hormones.

Nowadays, hypophysectomized rats may be obtained from certified suppliers who are licensed to perform the required operation, and who are experienced in postoperative handling and maintenance.

PROCEDURE

In the rat, the parapharyngeal approach is less destructive than the transauricular approach, and is preferred for bioassay. Male Wistar or Sprague-Dawley

rats weighing 110–150 g are anesthetized with modern anesthetics, and placed on a surgical table in a recumbent position. The head is tilted backwards to expose the operating field, the fur is removed with electric clippers, and the field of surgery is cleaned with alcohol. A median incision is made 2.5 cm in front of the sternum. The large salivary glands and the maxillary lymph nodes are retracted, the muscles over the trachea are divided at the midline, slightly below the thyroid a hole is made in the trachea (“tracheotomy”), and tracheal mucus is removed by vacuum aspiration. The insertion of a tracheal cannula is not necessary.

A deep blunt dissection is made directly medial to the tendon of the left or right digastric muscle. The ipsilateral glossopharyngeal nerve and the blood vessels are drawn to one side, and the pharynx and trachea are drawn to the other side, for an approach to the midline of the base of the skull. For the subsequent procedure, the use of an operating microscope is recommended (stereo microscope)

The base of the skull is cleaned using small pellets of cotton-wool. The spheno-occipital suture running between both mastoid processes is exposed. From behind, the crista occipitalis runs longitudinally up to the middle of this suture. Just at this point, a small hole is drilled at the base of the skull with a dental drill, taking care to leave the surrounding sinus blood vessels intact. The pituitary is now visible as a small, lens-shaped organ. With the tip of a fine glass tube, attached to a suction device, the pituitary gland is removed by aspiration. Occasional bleeding is stopped with a cotton-wool pellet. The trachea is now cleaned by suction pump and the wound closed. Animals are placed carefully in padded recovery cages, at an ambient temperature of 20–22°C for recovery. The removal of the posterior pituitary gland causes the animals to have experimentally induced diabetes insipidus and therefore they need access to sufficient quantities of drinking water.

MODIFICATIONS OF THE METHOD

Instead of a parapharyngeal approach, the **transauricular approach** may be used (S. Jung and H. G. Vogel unpublished data) to facilitate surgery. Male rats weighing 120–150 g are anesthetized and placed on their right side. The head is fixed between the thumb and the index finger of the left hand. In the perpendicular direction, a dental drill with a diameter of 2.2 mm is introduced into the meatus acusticus externus osseus. First, the fine lamellae of the middle ear are passed. Then, the drill is directed from the external ear to the contralateral orbita. In this way, the thin inner wall of

the bulla tympani is perforated. The junction of the bulla tympanica, os occipitale and os sphenoidale is located here. A small, straight forceps is introduced to remove bone fragments, and a blunt cannula diameter 1.6 mm is introduced to remove the pituitary gland by suction. The procedure needs much more technical skill than the current pharyngeal approach, and is more destructive.

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N.7.1

Gonadotropins

N.7.1.0.1

General Considerations

Selection of assay methods depends on the study's objectives, i. e., whether it is the biological activity of gonadotropins or the quantitative response of elements in the signaling chain being assessed. The biological assays have been widely replaced by methods for receptor binding and receptor-induced response (signaling).

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins which exist in multi-

ple molecular isoforms (microheterogeneity) with different molecular weights and with different degrees of glycosylation (Ulloa-Aguirre et al. 1988; Dahl and Stone 1991). The fine differences identified by analytical methods are not relevant for the bioassay, which measures the sum of biological activities. The immunological methods provide limited information on biological activity and need to be validated for detection of the intact hormone or cross-reactivity with inactive subunits. *In vivo* animal bioassays are available (e. g., the Steelman–Pohley assay), and *in vitro* cell bioassays with a high degree of sensitivity and specific radioreceptor assays have been developed (Simoni and Nieschlag 1991). Striking differences in assay results were found when comparing estimates obtained by *in vivo* bioassays, *in vitro* cell assays, receptor-binding assays, and immunoassays with the International Standard for Pituitary FSH, when assayed in terms of the Second International Reference Preparation of Human Pituitary FSH and LH (Storring and Gaines Das 1989).

For binding assays, gonadotropin receptors of various animal species have been cloned (Poyner and Hanley 1992). Time-resolved fluoroimmunoassay for gonadotropins has been proposed by Iwasawa et al. (1994). Application of methods for detection and quantification depends on the research objective, which may be targeted to physiological functions, or to the biochemical identification of isoforms and subunits (Imse et al. 1992).

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N.7.1.1**Follicle-Stimulating Hormone (FSH)**

Some of the methods described here are used for quantitative assay of biological FSH activity (in animals and *in vitro*); others are used for FSH activity in samples from nonclinical and clinical studies.

N.7.1.1.1**Ovarian Weight in hCG-Primed Rats****PURPOSE AND RATIONALE**

Follicle-stimulating hormone (FSH) increases the weight of ovaries in immature rats by inducing follicular maturation in a dose-related manner. This effect is greatly enhanced by simultaneous administration of a constant dose of human chorionic gonadotropin (hCG) for luteinization of the follicles, allowing the detection of low amounts of FSH (Steelman and Pohley 1953).

PROCEDURE

Immature female Sprague-Dawley rats weighing 40–45 g are treated for 3 days by subcutaneous injections of FSH (twice per day), using three doses of the standard (e. g., ovine NIH-FSH-S-9, National Institute of Health, Bethesda, Md., USA) or the test preparation (at the same dose interval). A constant amount of 25 IU hCG (Primogonyl, Schering, Berlin) is added. Hormone preparations are injected in physiological saline containing 2% gelatin. Six to eight animals are used per group. Then, 18 h after the last injection, the animals are sacrificed, then the ovaries are dissected out and weighed to the nearest 0.1 mg.

EVALUATION

Dose–response curves for standard and test compounds are plotted and potency ratios with confidence limits are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The Steelman–Pohley test is rather specific for FSH. The addition of luteinizing hormone (LH), thyroid-stimulating hormone (TSH), adrenocorticotropin (ACTH), human growth hormone (hGH), or prolactin did not influence the dose–response curves of FSH (Christiansen 1972b). The method has been adopted by pharmacopoeias (e. g., British Pharmacopoeia 1988).

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Evans et al. 1939; Brown 1955; Segaloff 1962; Parlow and Reichert 1963; Christiansen 1972a; Storrington and Gaines 1989

Igarashi and McCann (1964) reported a bioassay for FSH in mice using an added constant dose of hCG for augmentation, and uterine weight as the endpoint.

Brown and Wells (1966) described an assay of human urinary FSH with hCG augmentation, using ovarian weight in mice as the endpoint. The method has been used by Wide and Hobson (1986) to identify the effect of the assay method used to select the most active forms of FSH from human pituitary extracts.

Lamond and Bindon (1966) recommended the use of immature hypophysectomized mice for the assay of FSH with hCG augmentation, using uterine weight as endpoint.

Gans and van Rees (1966) studied a testicular augmentation assay method for FSH. Immature male rats were hypophysectomized and on the same day the right testis was removed and weighed. Treatment with various doses of FSH together with a constant dose of 20 IU hCG was started on the next day and continued for 6 days. On the 7th day, the animals were sacrificed and the remaining left testis was removed and weighed. The difference between weights of the right and left testis in each animal was used as the endpoint.

Uberoi and Meyer (1967) used the uterine weight of the immature rat as a pituitary gonadotropin assay, with augmentation by hCG.

Results obtained with the *in vivo* bioassay (Steelman and Pohley 1953) have been compared with an *in vitro* bioassay based on estradiol production by cultured Sertoli cells, and with an FSH immunoassay by Storrington et al. (1981).

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N.7.1.1.2

[³H]Thymidine Uptake in Cultured Mouse Ovaries

PURPOSE AND RATIONALE

Follicle-stimulating hormone stimulates [³H]thymidine uptake by cultured mouse ovaries in a dose-dependent manner, using the tissue-specific proliferation response of the follicles (Ryle 1971; Boggins and Ryle 1972).

PROCEDURE

Intact ovaries are obtained from 15-day-old mice. They are dissected carefully with the aid of a stereomicroscope and transferred to culture dishes. Each ovary is placed on a strip of lens tissue supported on a stainless steel mesh grid 4 mm above the floor of a plastic Petri dish and incubated in Eagle's medium supplemented with glucose and glutamine. The dishes are gassed with 5% CO₂ in air at 37°C. Three replicate dishes are used for each concentration of the standard (0.1 and 0.4 IU/ml) and of the test preparation. [³H]Thymidine (0.02 μC) is added to each dish the day after the cultures are set up. Three days later the tissue is prepared for counting. Each grid is irrigated with about 5 ml saline solution. The ovary is then transferred to a counting vial and dissolved for counting the incorporated radioactivity in a liquid scintillation counter.

EVALUATION

Dose–response curves of [³H]thymidine uptake are established for the standard and the test preparation, and potency ratios with confidence limits are calculated. Advantages over animal bioassays include increased specificity and a reduced requirement for a purified test preparation, which may be available only in small quantities.

CRITICAL ASSESSMENT OF THE METHOD

The [³H]thymidine uptake by cultured mouse ovaries has found less acceptance than other *in vitro* methods described below.

MODIFICATIONS OF THE METHOD

Follicle cultures from mouse ovaries for the study of follicular metabolism have been described by Boland et al. (1993).

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N.7.1.1.3

Granulosa Cell Aromatase Assay in Vitro

PURPOSE AND RATIONALE

The granulosa cell aromatase assay *in vitro* is based on the principle of the *in vivo* bioassay of Steelman and Pohley (1953), in which the ovarian weight response is the result of FSH-induced follicular maturation and induced estradiol production, which stimulates ovarian growth. The co-administration of human chorionic gonadotropin (hCG) at a standard amount enhances androgen production by theca cells, which in turn provides the androgen substrate for FSH-dependent aromatase activity in granulosa cells. Hsueh et al. (1983) and Jia and Hsueh (1985) developed a sensitive *in vitro* bioassay for FSH based on the stimulation of estrogen production by cultured granulosa cells in serum-free medium containing androstenedione.

PROCEDURE

Intact Sprague-Dawley rats (21–22 days old) are implanted with Silastic capsules containing diethylstilbestrol to stimulate granulosa cell proliferation. Four

days after implantation, the animals are sacrificed and ovaries are dissected for granulosa cell collection. The ovaries are decapsulated, follicles are punctured with 27-gauge hypodermic needles, and granulosa cells are carefully expressed into McCoy's 5a medium. An aliquot is diluted with trypan blue stain, and viable cells are counted with a hemocytometer. Cells are cultured in 16-mm, 24-well culture plates for 2–3 days at 37°C in a humidified, 95% air–5% CO₂ incubator. Each well contains 5 × 10⁴ viable cells in 0.5 ml McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 10⁻⁷ M diethylstilbestrol (DES), 10⁻⁶ M androstenedione (as aromatase substrate), 0.125 mM 1-methy-3-isobutylxanthine (MIX), 1 µg/ml insulin, and 30 ng/ml hCG.

For the measurement of FSH bioactivity in serum samples, each test is performed in triplicate at three dose levels (5, 10, and 20 µl). To ensure a constant volume of 20 µl serum in the total incubation volume of 500 µl, gonadotropin-free serum is added as required. For the FSH standard curve, 4% gonadotropin-free serum is added to the culture medium. The sensitivity is increased when the serum is pretreated with 12% polyethylene-glycol (PEG, mol. wt. 8000). After a culture period of 3 days, estradiol content in the medium is measured by radioimmunoassay (RIA).

EVALUATION

RIA data are analyzed using weighted logit-log regression analysis. Calculation of FSH bioactivity in serum samples or test preparations is performed using a standard curve fitted with a second-order polynomial.

MODIFICATIONS OF THE METHOD

Dorrington et al. 1975; Hsueh et al. 1984; Jia and Hsueh 1986; Dahl et al. 1989; Fauser et al. 1989; Storrington and Gaines 1989; Matzkin et al. 1990; Simoni and Nieschlag 1991; YoungLai et al. 1992

Sensitivity may be improved by using a specific response of the follicles. Beers and Strickland (1978), Wang and Leung (1983), Combarous et al. (1984), and Thakur et al. (1990) found that FSH led to a dose-dependent increase of plasminogen activator production in cultures of rat granulosa cells. This assay has been found to be extremely sensitive: as little as 10⁻¹⁵ mol of FSH could be detected.

Ax and Ryan (1979) found that FSH dose-dependently stimulates ³H-glucosamine incorporation into proteoglycans by porcine granulosa cells *in vitro*.

Bhargava et al. (1989) used normal human ovarian cells for long-term cultures. These cells produced

progesterone from cholesterol or pregnenolone and estrone from androstenedione when stimulated by FSH.

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N.7.1.1.4

Sertoli Cell Aromatase Assay in Vitro

PURPOSE AND RATIONALE

The Sertoli cell aromatase assay was first described by Van Damme et al. (1979). This *in vitro* bioassay was developed following the observation by Dorrington et al. (1975) that FSH, but not LH, stimulates estradiol production by cultured Sertoli cells from immature rats. The assay has been further improved by Ritzén et al. (1982), Shah and Ritzén (1984), and Padmanabhan et al. (1987). These assays are used to determine bioactive FSH in serum for diagnostic use, and they are also applicable for measuring the activity of FSH preparations intended for therapy.

PROCEDURE

Sertoli cells are obtained from 7- to 10-day-old male Sprague-Dawley rats. Testes are decapsulated, the testicular tissue is chopped twice, at right angles, with a mechanical tissue slicer and incubated in culture medium containing 0.03% collagenase and 0.003% trypsin inhibitor for 5–10 min at 34°C. After the initial dispersal, seminiferous tubules settle to the bottom of the incubation flask, and the medium is decanted to remove interstitial cells. The tubules are washed several times; resuspended in medium that contains collagenase, trypsin inhibitor and 0.03% DNase (to prevent clumping of cells), and then incubated for 30 min at 34°C. During incubation, dispersion of Sertoli cells is hastened by repeated aspiration with a Pasteur pipette. The resulting cell suspension is washed three times; cell number and viability are determined by counting in trypan-blue-dye-containing medium. Cells are suspended in a density of 5×10^5 viable cells/ml. One milliliter of cell suspension is transferred to each well of Falcon multiwell culture dishes (16 mm diameter, Falcon Plastics, Oxnard, Calif., USA).

Sertoli cells are cultured in medium comprised of the following constituents: 1:1 (vol/vol) mixture of Ham's F-10 nutrient mixture and Dulbecco's Modified Eagle's Medium that contains 1.2 g/l sodium bicarbonate, 20 mg/l gentamicin, and 1 mg/l amphotericin. Also included in the medium are: 1 µg/ml insulin, 5 µg/ml transferrin, 10 ng/ml epidermal growth factor, 20 pg/ml T₄, 10⁻⁸ M hydrocortisone, and 10⁻⁶ M retinoic acid. The cell cultures are incubated in this medium in a water-saturated atmosphere that consists of 95% air and 5% CO₂ at 37°C.

After an initial incubation of the cell monolayers for 72 h, the medium is removed; the cells are washed once with the medium and re-incubated in the medium described above, which contains 2.5×10^{-6} M 19-hydroxyandrostenedione, FSH standard or unknown samples at various concentrations, and 0.1 mM methylisobutylxanthine (MIX). After a 24-h incubation, the medium is aspirated and centrifuged. The resultant supernatants are stored frozen until estradiol measurement by radioimmunoassay (England et al. 1974).

EVALUATION

The results are expressed as picograms of estradiol formed per milliliter culture medium. To evaluate changes in estradiol secretion with changes in FSH concentration, regression analyses are performed. From these data, activity ratios with confidence limits are calculated.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Dorrington and Armstrong 1975; Marana et al. 1974; Storrington et al. 1981; Zaidi et al. 1981; Foulds and Robertson 1983; Wide and Hobsen 1983, 1986; Khna et al. 1984; Harlin et al. 1988; Storrington and Gaines 1989; Simoni and Nischlag 1991;

Rao and Ramachandran (1975) used isolated rat seminiferous tubule cell preparations free of Leydig cells for determination of cyclic AMP production as an *in vitro* assay for FSH.

Sairam and Manjunath (1982) used rat seminiferous tubular suspensions containing Sertoli cells, incubated them with FSH preparations and measured cyclic AMP formation as the endpoint.

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- have been found by Marana et al. (1979), Zaidi et al. (1981), Foulds and Robertson (1983), and Burgon et al. (1993). This is attributed to measuring binding activity, which may differ from subsequent intracellular signaling. Several receptor binding assays have been described [Cheng (1975); Andersen et al. (1983) using bovine testes; Reichert (1976) using rat testes tubule tissue].

PROCEDURE

Membrane preparations from bovine testes are used according to the Cheng (1975) and Andersen et al. (1983). Fresh bovine testes or testes from rats weighing 220–280 g are decapsulated and rinsed with cold 0.025 M Tris-HCl buffer at pH 7.2, containing 0.3M sucrose, and then minced and homogenized with a Polytron homogenizer at maximum speed for 30 s at a concentration of 5 ml buffer per gram of tissue. The homogenate is first filtered through four layers, and the filtrate is again filtered through eight layers of cheesecloth. The filtrate is then centrifuged at 12,000 g for 30 min at 4°C. The pellet is discarded and the supernatant is further centrifuged at 100,000 g for 1 h at 4°C. The supernatant is discarded and the pellet resuspended in cold 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM MgCl₂, at a concentration of 1 ml buffer per gram of the original weight of the testis. The isolated membranes are stored at –70°C in aliquots of 10 ml per vial until use.

For assays, 12/75 mm glass disposable tubes are used. To each tube, 0.2 ml of 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM MgCl₂ and 0.1% BSA, 0.1 ml of standard FSH or unknown samples in the same buffer, 0.1 ml of ¹²⁵I-hFSH tracer labeled by the lactoperoxidase method (50,000 cpm, approximately 2 ng), and finally 0.1 ml of plasma membrane receptors of appropriate dilution (approximately 1–2 mg/ml) are added to reach a final volume of 500 µl per tube. All the above solutions are kept at 4°C before use. The tubes are then shaken vigorously and incubated at room temperature for 20 h. Following incubation, the reaction is stopped by adding 3.0 ml of cold 0.025 M Tris-HCl buffer containing 0.1% BSA. After centrifugation at 4000 rpm for 30 min, the supernatant is drained and the tip of each tube is dried. The pellet remaining at the bottom of the tube is counted in an automatic gamma counter.

EVALUATION

Specific binding (%) is defined as $(C_B - C_N) \times 100 / C_T$, where C_B is the cpm bound to the testicular receptor (pellet), C_N is the nonspecific bound cpm (not

N.7.1.1.5

Receptor Binding Assay for FSH

PURPOSE AND RATIONALE

Significant differences between the receptor binding activity of FSH preparations and biological activity

displaced by 1000-fold excess of unlabeled hFSH), and C_T is the total cpm added per tube. For standard curves, the specific binding of tracer ^{125}I -hFSH in the absence of cold hormone (6%–8% by 150–200 μg of receptor protein) is taken as 100% bound ^{125}I -hFSH, and the nonspecific bound ^{125}I -hFSH (1%–2% of the total cpm added) as the baseline. The specific binding of ^{125}I -hFSH over a range of hFSH concentrations is used for calculating a standard curve and sample concentrations.

MODIFICATIONS OF THE METHOD

The method has been used by several authors: Lee and Ryan 1973; Schwartz et al. 1973; Ketelslegers and Catt 1974; Reichert and Bhalla 1974; Calvo et. al 1986; Storrington and Gaines 1989

Simoni et al. (1993a) analyzed the biological and immunological properties of the International Standard for FSH 83/575 by isoelectrofocusing and made a comparison with other FSH preparations. The results suggested that the international standard 83/575 is not fully representative of pituitary and serum FSH, and its use for calibration of immunometric methods based on monoclonal antibodies is unlikely to resolve problems of inaccuracy in the measurement of serum FSH.

Grasso et al. (1993) studied the effects of a testicular toxicant on FSH binding to membranes of cultured rat Sertoli cells, as a method in toxicology.

Wakabayashi et al. (1997) reported the cDNA cloning and transient expression of a chicken gene encoding an FSH receptor.

Simoni et al. (1993b) analyzed the molecular heterogeneity of two batches of commercially available urofollitropin by immunofluorometric assay, radioligand receptor assay and *in vitro* bioassay.

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N.7.1.2

Luteinizing Hormone (LH) = Interstitial Cell Stimulating Hormone (ICSH)

Some of the methods described here are classical animal bioassays for LH/ICSH preparations, others are *in vitro* bioassays used in research and for clinical samples.

N.7.1.2.1

Prostate Weight in Hypophysectomized Rats

PURPOSE AND RATIONALE

The assay is based on a biological response. Injection of luteinizing hormone (ICSH) in hypophysectomized

male rats causes enlargement of the testes and the secondary sexual organs. The interstitial cells of the testes (Leydig cells) are stimulated by LH/ICSH and secrete androgens which in turn stimulate the accessory organs, such as the ventral prostate of the rat.

PROCEDURE

The method has been used by several authors: Greep et al. 1942; Segaloff et al. 1956; Segaloff 1962; British Pharmacopoeia 1988;

Immature male Sprague-Dawley rats aged 21 days are hypophysectomized. From the 2nd to the 5th day, they receive daily subcutaneous injections of two or more doses of the test preparation and the LH/ICSH standard. They are sacrificed on the 6th day. Both testes and the ventral prostate are prepared and weighed. LH/ICSH – but not FSH – induces a specific dose-dependent increase of prostate weight, whereas FSH also induces an increase of testis weight. A predominant LH activity in the test material mainly stimulates prostate weight, but not testis weight.

EVALUATION

Dose–response curves are plotted for the ventral prostate of each group of test preparation or standard allowing the calculation of activities and potency ratios with confidence limits.

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N.7.1.2.2

Superovulation in Immature Rats

PURPOSE AND RATIONALE

An assay for evaluation of luteinizing hormone (LH) and the luteinizing component of human chorionic gonadotropin (hCG) was described by Zarrow et al. (1958). The sensitivity of the response is enhanced by priming with pregnant mares' serum gonadotropin (PMSG – mainly follicle-stimulating hormone activity) and by intravenous injection of the LH test preparation.

PROCEDURE

Groups of five immature female rats (Charles River strain, age 21 days) are treated with a priming dose of 30 IU of PMSG to induce follicular development. Increasing doses of LH reference or test preparations are injected intravenously 56 h later, to induce ovulation. Then 24 h after the final injection, the animals are sacrificed, the fallopian tubes are removed and examined under a dissecting microscope (magnification of 40×) ϕ for the presence oocytes (ova). Ovulation is easily noted by an enlarged translucent segment of the tube, through which the ova can be seen. The segment is punctured with a dissecting needle and the entire cumulus clot containing the ova is expelled. The clump containing the oocytes is treated with hyaluronidase, and the number of ova is counted.

EVALUATION

Doses–response curves are obtained using three doses of standard/reference and test preparation for the calculation of potency ratios with confidence limits.

REFERENCES AND FURTHER READING

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N.7.1.2.3

Ascorbic Acid Depletion of Ovaries in PMSG/hCG-Primed Rats

PURPOSE AND RATIONALE

Luteinizing hormone (LH, also known as interstitial cell stimulating hormone or ICSH) induces dose-dependent depletion of ascorbic acid in the ovaries of rats primed with pregnant mares' serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (Parlow 1961; Parlow and Reichert 1963; Sandow et al. 1972).

PROCEDURE

Female Sprague-Dawley rats weighing 40 ± 5 g are injected with 50 IU PMSG (e.g., Anteron, Schering, Berlin) in 0.2 ml saline subcutaneously on day 1 at 3:00 p.m. On day 3, the rats receive 25 IU hCG (e.g., Primogonyl, Schering, Berlin) subcutaneously at 9:00 a.m. On day 7, three different doses of the standard (e.g., NIH LH-S-1, National Institute of Health, Bethesda, Md., USA) and the test substance are injected subcutaneously. Eight animals are used per group. Three hours later, the animals are sacrificed,

and both ovaries are removed, weighed, and homogenized for determination of ascorbic acid content.

EVALUATION

Dose–response curves for standard and test compound are plotted and activity ratios with confidence limits calculated.

REFERENCES AND FURTHER READING

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N.7.1.2.4

Testosterone Production by Leydig Cells in Vitro Induced by LH

PURPOSE AND RATIONALE

Luteinizing hormone (LH) increases the biosynthesis and secretion of androgens in the Leydig cells of the testes. This can be used for an *in vitro* assay using isolated Leydig cells.

PROCEDURE

For Leydig cell isolation, male Wistar or Sprague-Dawley rats weighing 250–300 g are sacrificed, the testes removed and immediately decapsulated. Two testes are then digested with 7 ml of collagenase solution (1 mg collagenase/1 ml Gibco medium 199) at 37°C for 18 min. Plastic tubes of 40 ml capacity with tight-fitting caps are used, and are placed longitudinally in the water bath with constant shaking (75 cycles/min). After incubation, 15 ml of cold saline is added to each tube. The tubes are inverted a few times and then left at 4°C for 10 min. The supernatants are then carefully siphoned off from the top. The clear supernatant is mixed with an equal volume of 26% Ficoll/0.4% BSA/medium 199 at pH 6.5, and then centrifuged at 1500 g for 10 min at 4°C. The cell pellet obtained from the centrifugation is suspended in a known volume of incubation medium (Medium 199 containing 1% BSA and 0.01% lima bean trypsin inhibitor). An aliquot of the cell suspension is taken for counting in a Coulter counter, and the balance is diluted with the incubation medium giving a density of 4×10^6 cells/ml.

For each assay, 0.25 ml of the cell suspension is used. The assay is carried out in 12 × 75 mm plastic tubes incubated with graded doses of test compound at 37°C. Constant shaking (100 cycles/min) under a 95% O₂/5% CO₂ atmosphere for 3 h is required. At the end of the incubation, each tube is centrifuged, and the supernatant is assayed for testosterone by a radioimmunoassay (Dufau et al. 1972). The assay is carried out in triplicate and needs to be repeated at least once with different batches of donor animals.

EVALUATION

Dose–response curves are obtained with various doses of standard and test preparation to allow calculation of activities and potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Van Damme et al. 1974; Dufau et al. 1976; Janszen et al. 1976; Dufau et al. 1977; Khan et al. 1984; Liu et al. 1984; Harlin et al. 1988; Stadler et al. 1989; Haavisto et al. 1990; Rodgers et al. 1992

The effect of chemically deglycosylated human chorionic gonadotropin on cAMP and testosterone production in rat Leydig cells has been used by Chen et al. (1982) for characterization.

Cultures of mouse Leydig cell tumor cells (MA10) have been used by Ascoli (1981), Whitcomb and Schneyer (1990), and Dahl and Sarkissian (1993).

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N.7.1.2.5

Receptor Binding Assay for LH

PURPOSE AND RATIONALE

LH receptors are membrane receptors that mediate the initial hormone binding step; they are suitable for structure–activity studies and *ex vivo* analysis of receptor concentration changes. Luteinizing hormone is a glycoprotein composed of an α - and a β -subunit. Carbohydrate moieties are attached to both. Removal of the carbohydrate chains reduces bioactivity but enhances receptor binding.

PROCEDURE

For the preparation of Leydig cell receptors, the partially purified Leydig cells obtained from Ficoll gradient centrifugation as described above for the testosterone formation test are used. The cell pellet obtained from the Ficoll gradient centrifugation is first washed twice with unlabeled radioligand buffer (0.1 M Tris-HCl, pH 7.4, containing 5 mM MgCl₂, 0.1 M sucrose, and 0.1% BSA). The pellet is resuspended in a known volume of the same buffer. An aliquot of this suspen-

sion is taken for cell counting in a Coulter counter, and the balance is homogenized in a Polytron homogenizer for 10 s to break up the cells. This prevents internalization of the hormone molecule during the binding assay. The iodination of LH is carried out according to the chloramine-T method (Liu et al. 1977). After the addition of 2.5 ng of ¹²⁵I-labeled LH tracer, the binding assay is carried out at 37°C for 2 h with constant shaking. The hormone–receptor complex is then separated by centrifugation and bound radioactivity is counted in a Packard gamma-counter.

EVALUATION

Competitive–inhibition curves for specific binding are established and binding capacity is calculated.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Liu et al. 1974; Catt et al. 1976; Liu et al. 1984

Lee and Ryan (1972) described specific binding of human luteinizing hormone (LH) to homogenates of luteinized rat ovaries.

Storring and Gaines-Das (1993) described the second International Standard for Human Pituitary LH. Two new batches (coded as 80/552 and 81/535) were compared with the International Reference Preparation of Human Pituitary LH for Immunoassay (IRP 68/40) by 19 laboratories in 11 countries, using *in vivo* and *in vitro* bioassays, a receptor assay and immunoassays. An activity of 35 International Units of Human Pituitary LH was assigned to the contents of each ampoule coded 80/552.

Selvaraj and Moudgal (1993) used sheep luteal membranes for an LH receptor assay capable of measuring serum LH/chorionic gonadotropin (CG) in a wide variety of species.

Chen and Bahl (1993) reported a high expression of the hormone-binding active extracellular domain (1–294) of rat lutropin receptor in *Escherichia coli*.

Jia et al. (1993) developed a LH/CG bioassay using 293 cells permanently transfected with the human LH receptor cDNA and a luciferase reporter gene driven by a cAMP-dependent promoter.

Selvaraj et al. (1996) established a radioreceptor assay for LH/CG in human sera using immortalized granulosa cells transfected with LH/CG receptor.

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N.7.1.3

Other Gonadotropins

GENERAL CONSIDERATIONS

The pituitary gonadotropins are species specific and exist in various isoforms containing different carbohydrate moieties. In the treatment of infertility, they were substituted by gonadotropin preparations from human urine purified by extraction (human menopausal gonadotropin, hMG, and human chorionic gonadotropin, hCG, from urine in pregnancy), containing variable proportions of follicle-stimulating and luteinizing activity. Pituitary gonadotropins for the treatment of infertility are now obtained by biosynthesis (recombinant DNA methods). Their structure closely resembles human FSH and LH.

Human chorionic gonadotropin (hCG) is excreted in the urine of pregnant women, and may also be excreted by tumors, such as hydatidiform mole and chorionepithelioma. hCG is measured as a tumor marker, and for the diagnosis of early pregnancy. hCG has mainly LH-like activity. Purified preparations of hCG and hMG are tested by bioassays.

Human menopausal gonadotropin (hMG) is excreted in the urine of postmenopausal women. hMG

has predominantly FSH-like activity and was used to induce follicular maturation.

A gonadotropin preparation previously used in therapy is *pregnant mares' serum gonadotropin (PMSG)*. Pregnant horses secrete large amounts of pituitary gonadotropin, which is not excreted in the urine and accumulates in serum. PMSG has predominantly FSH-like activity.

N.7.1.3.1

Corpus Luteum Formation in Immature Mice (Aschheim–Zondek Test)

PURPOSE AND RATIONALE

Aschheim and Zondek (1927, 1935) injected the urine of pregnant women (containing FSH and LH activity) into immature female mice and observed the ovulation and formation of corpora lutea. This procedure was widely used as a biological pregnancy test and later replaced the hCG assays. Further studies were performed by Hamburger and Pedersen-Bjergaard (1937).

PROCEDURE

Groups of 10–20 mice, 21 days of age, are treated with 5 equal subcutaneous injections of urinary extracts over the course of 48 h. The animals are sacrificed 96 h after the first injection. The ovaries are dissected and the formation of corpora lutea is observed by examination with a lens or a stereomicroscope. Rupture of follicles is indicated by blood spots.

EVALUATION

The number of ovaries showing the formation of corpora lutea was expressed as dose–response curves.

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N.7.1.3.2

Biological Assay of hCG in Immature Male Rats

PURPOSE AND RATIONALE

The LH-like activity of hCG can be determined by measuring the weight increase of the prostate and seminal vesicles in immature male rats.

PROCEDURE

Immature male Sprague-Dawley rats at an age between 21 and 24 days are assigned at random to six groups of at least five animals. Three doses of standard (e.g., 4, 8, and 16 IU) and corresponding doses of the test preparation are dissolved in albumin-phosphate buffer, pH 7.2, and injected subcutaneously daily over a period of 4 days. On the 5th day, the animals are sacrificed and the seminal vesicles and prostate glands are prepared and weighed.

EVALUATION

Dose-response curves of the weights of seminal vesicles and ventral prostate glands are established for standard and test preparations allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

The United States Pharmacopoeia USP 23 (1995) requests standardization of hCG in immature female rats at an age of 20–23 days. The animals are injected subcutaneously with three different doses of test preparation or standard daily on 3 days consecutively. They are sacrificed on the 5th day and the uteri are prepared and weighed.

REFERENCES AND FURTHER READING

- British Pharmacopoeia (1988) Biological assay of chorionic gonadotrophin. Appendix XIV C. HMSO, London, pp A164–A165
- United States Pharmacopoeia USP 23 (1995) Chorionic gonadotropin. United States Pharmacopoeial Convention, Rockville, Md., pp 718–719

N.7.1.3.3**Receptor Binding Assay for hCG****PURPOSE AND RATIONALE**

The initial binding step to the luteinizing hormone (LH) receptor is used for quantification; the receptors may be obtained from superovulated rat ovaries, or from other tissues. Selvaraj et al. (1996) described an *in vitro* bioassay and radioreceptor assay for LH/chorionic gonadotropin (CG) in human sera using immortalized granulosa cells transfected with the LH/CG receptor.

PROCEDURE

Ovaries from pseudopregnant rats primed with pregnant mares' serum gonadotropin (PMSG) and hCG are homogenized. The homogenates are centrifuged at 2000g for 15 min and the pellets are washed

3 times with 40 mM Tris buffer and resuspended in the same buffer. The suspension is filtered through four layers of cheesecloth prior to use. Fresh 2000-g fraction is prepared for each experiment. Purified hCG is labeled by the chloramine-T method resulting in a specific activity of approximately 60–70 $\mu\text{Ci}/\mu\text{g}$.

For assessment of binding to receptors, a mixture consisting of the 2000-g fraction (equivalent to 2.5 or 5 mg of wet ovary or 45–90 μg of protein) and $(0.1\text{--}30) \times 10^{-10}$ M labeled gonadotropin or other test substances is incubated in a final volume of 1 ml of 40 mM Tris buffer (pH 7.4) containing 0.1% BSA at 25°C for 16 h. Then 1 ml ice-cold Tris buffer is added to the medium. This is immediately filtered, with suction, through Millipore EHWP filters (pore size 0.5 μm) previously wetted with 4% BSA to reduce nonspecific binding. The adsorbed material is washed with another 10 ml of ice-cold Tris buffer. The radioactivity on the filter is measured. Binding in the presence of a large excess of unlabeled hCG (200 IU/ml) is used to assess nonspecific binding.

EVALUATION

Specific binding is calculated and dose-response curves are obtained.

MODIFICATIONS OF THE METHOD

Similar studies were reported by: Catt et al. 1972, 1976; Lee and Ryan 1973; Saxena 1976; Keutmann et al. 1983.

REFERENCES AND FURTHER READING

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N.7.1.4**Human Menopausal Gonadotropin (hMG)****N.7.1.4.1****Biological Assay of hMG in Immature Rats****PURPOSE AND RATIONALE**

The FSH-like activity of hMG can be measured by the ovarian weight response of immature rats. The LH-like activity of hMG can be determined in immature male rats by the increase of prostate and seminal vesicle weights.

PROCEDURE

Female Sprague-Dawley rats at an age between 21 and 24 days are assigned at random to six groups of at least five animals. Three doses of standard (e. g., a total dose of 1.5, 3, and 6 IU) and corresponding doses of the test preparation are dissolved in albumin-phosphate buffer, pH 7.2, and injected subcutaneously daily over a period of 3 days. On the 4th day, the animals are sacrificed and the ovaries are removed and weighed.

EVALUATION

Dose-response curves for FSH-like activity are established, and potency ratios with confidence limits may be calculated using hMG standard preparations.

REFERENCES AND FURTHER READING

British Pharmacopoeia (1988) Biological assay of menotrophin. Follicle-stimulating activity. Appendix XIV C. HMSO, London, pp A165–A166

N.7.1.5**Pregnant Mares' Serum Gonadotropin (PMSG)****N.7.1.5.1****Biological Assay of PMSG in Immature Female Rats****PURPOSE AND RATIONALE**

The predominant FSH-like activity of equine gonadotropin preparations is determined by the ovarian weight response of immature rats, as described for hMG (Hamburger 1950).

REFERENCES AND FURTHER READING

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N.7.1.6**Immunoassays of Gonadotropins**

Immunoassays of gonadotropins are important in clinical diagnostics (RIA, ELISA and related methods). For pharmacological and biological experiments, they are measured in blood, urine, and pituitary tissue, e. g., for evaluation of gonadotropin releasing hormones (as described in Sects. N.9.2.5 and N.9.2.6). Many comparisons for results of *in vivo* and *in vitro* bioassays versus immunoassays showed significant discrepancies when expressed as the ratios of bioactive to immunoreactive LH (B/I ratio). However, there are numerous validated species-specific methods which are used for experimental endocrinology and clinical diagnosis.

Similar studies were reported by: Faiman and Ryan 1967; Wide and Hobson 1983; Ulloa-Aguirre et al. 1988; Seth et al. 1989; Terouanne et al. 1989; Armbruster and Haws 1990; Wheeler 1991; Rosenfield and Helke 1992; Weiss et al. 1992; YoungLai et al. 1992; Haavisto et. al 1993.

REFERENCES AND FURTHER READING

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N.7.1.7

Gonadotropin inhibition

N.7.1.7.1

General Considerations

Gonadotropin inhibition is suspected when, in early testing of compounds or in toxicology studies, weight reduction of gonads and the endocrine-dependent organs is found. Early tests used the physiological feedback effects of endogenous steroids in rats, e. g., the parabiosis experiment. This technique in rats was applied to the relationship between central and peripheral endocrine organs, the pituitary–gonadal axis. Basically, the deficit in gonadal steroid hormones after castration of one parabiotic partner induces increased secretion of gonadotropin which in turn stimulates hypertrophy of the gonads of the noncastrated partner. The procedure is described in detail by Byrnes and Meyer (1951); Shipley (1962). The parabiosis technique is now obsolete but was applied again a few years ago with remarkable success in the identification of the adipose tissue hormone leptin (see Sect. L.4.1).

REFERENCES AND FURTHER READING

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N.7.1.7.2

Inhibition of Gonadotropin Secretion in Intact Animals

PURPOSE AND RATIONALE

The suppression of gonadotropin secretion by steroid compounds (as an example) can be detected in the endocrine survey test, described in Sect. N.1. Young rats, e. g., 80–100 g initial body weight, are treated with gonadal steroids or their derivatives to induce gonadotropin suppression by negative feedback of steroids (McGinty and Djerassi 1953; Saunders and Drill 1958; Shipley 1962). In intact rats, a reduction of gonadal weight indicates gonadotropin inhibition, whereas in castrated control groups (females or males), the weight increase of the sexual-hormone-dependent organs indicates the intrinsic estrogenic or androgenic property of the test compound.

PROCEDURE

Immature male and female Sprague-Dawley rats weighing 55–65 g or young rats 80–100 g are used. Groups of ten animals per sex and dosage group are treated daily over a period of 21 days with doses of the test compound usually by the oral or subcutaneous route. Controls receive the vehicle only. Testosterone may be used as reference compound (standard) in males and estradiol as the reference compound in females. Twenty-four hours after the last treatment, the animals are sacrificed and body weights recorded: in males, testes, seminal vesicles, ventral prostate and musculus levator ani; and in females, ovaries and uterus are dissected out and weighed. In addition, the gonadotropin content of the pituitary glands at autopsy may be determined to detect inhibition of gonadotropin synthesis.

EVALUATION

Organ weight averages are calculated for each treatment group and compared with controls. The reference steroids testosterone and estradiol suppress gonadal weight by pituitary gonadotropin inhibition (negative feedback) in intact animals, and increase gonadal weight in castrated animals by direct action. Dose–response curves can be established for the test compounds and the standard, and the relative potency of gonadotropin inhibition is calculated.

MODIFICATIONS OF THE METHOD

As an example of a classical bioassay, gonadotropin inhibition can be tested in semicastrated male rats (H. G. Vogel unpublished data, 1964). Male Sprague-Dawley rats weighing 50–60 g are orchietomized on the left side. The testis is weighed (without epididymis and epididymal fat). Test compounds in various doses or the standard are administered subcutaneously once daily for a period of 10 days. Controls receive the vehicle only. Standard compounds may be medroxyprogesterone acetate 0.4, 2.0, and 10.0 mg per animal per day, or 17-ethinyl-19-nortestosterone acetate 0.1, 0.5, and 2.0 mg per animal per day subcutaneously.

On day 11, the animals are sacrificed and the remaining testis, the adrenals, the seminal vesicles, the ventral prostate and the musculus levator ani are dissected out and weighed. Gonadotropin suppression results in atrophy of the remaining (contralateral) testis as compared with testis weight of the untreated control. A decrease in adrenal weight indicates feedback inhibition of the pituitary–adrenal axis. The weight of seminal vesicles, the ventral prostate and the musculus levator ani indicates androgenic activity of the test

compound. Dose–response curves for the test compound and the standard as well as potency ratios are calculated.

REFERENCES AND FURTHER READING

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N.7.1.7.3

Inhibition of Ovulation and Luteinization

PURPOSE AND RATIONALE

These methods were used frequently in research on contraception and selection of contraceptive steroids. Described here is an assay for progestational steroids. Progestagens inhibit pituitary gonadotropin secretion and synthesis. Progestational compounds, e. g., cyproterone acetate, inhibit ovulation and corpus lutea formation in young female rats by reducing luteinizing hormone secretion.

PROCEDURE

Female rats (35 days old) are treated once daily over a period of 7 days with increasing doses of gestagens, e. g., 0.125–2.0 mg progesterone or 0.025–0.40 mg 6 α -methyl-17 α -acetoxy-progesterone, subcutaneously. Controls receive the vehicle only. On the 8th day, the animals are sacrificed and body weight, ovarian weight, and number of corpora lutea are recorded. Progestogens inhibit follicular maturation, ovulation, and corpus lutea formation in this assay.

EVALUATION

Dose–response curves are established for average number of corpora lutea per animal and ovarian weight.

Similar studies were reported by: Junkmann 1957; Shibley 1962; Hebborn 1971; Phillips et al. 1987; Uilenbroek 1991

MODIFICATION OF THE METHOD

Ovum count can be performed in the oviduct of immature mice or rats after treatment with low doses of estradiol (Austin and Bruce 1956; May 1971). Immature rats or mice are injected with estradiol or stilbestrol at several dose levels to initiate estrus and ovu-

lation. Then 72 h later the animals are sacrificed, and the oviducts are removed and examined under a stereomicroscope for the presence of ova. They can be seen through the swollen translucent walls of the oviduct. The swollen part of the oviduct is punctured with dissecting needles to release the ova and count the number of ova per animal. The rate of ovulation (number of ova) is related to the dose of estrogenic compound. In a modification of the method, inhibition of the rate of ovulation induced using a standard dose of estradiol by anti-ovulatory test compounds may be determined.

Hahn et al. (1977) treated rats in diestrus with test compounds administered orally in 0.5 ml sesame oil, and again on the following day when the animals were in proestrus. The animals were sacrificed on the next day, at which time they would normally have ova in the proximal segment of the fallopian tubes. Oviducts were separately flushed with saline onto a glass microscope slide, and the condition and number of ova were noted.

Inhibition of ovulation by gestagens can be studied in rabbits (Shibley 1965). Progesterone has a time-dependent effect on ovulation (stimulating or inhibiting secretion of pituitary gonadotropins). The timing of progesterone injection relative to the anticipated time of ovulation is important. Progesterone injected less than 4 h before ovulation in rabbits facilitates ovulation. In contrast, when injected more than 4 h before the anticipated time of ovulation (Sawyer 1952), progesterone inhibits ovulation. The rabbit ovulates within a few hours after mating, after mechanical stimulation of the vagina or after an intravenous injection of copper acetate (0.3 mg/kg). Progesterone injected 24 h before induction of ovulation will prevent ovulation.

Sexually mature female rabbits weighing 3–4 kg are treated with various doses of a standard progestogen or the test compound and 24 h later an ovulation-inducing stimulus is given. The rabbits are sacrificed and the ovaries are examined 18–24 h later. The total number of ovulation points on both of the ovaries is recorded for each animal. Dose–response curves for standard and test preparations are calculated.

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N.7.1.7.4

Ovary–Spleen Transplantation

PURPOSE AND RATIONALE

This is a historical assay for gonadal steroids. Venous outflow from the spleen is exclusively to the liver. The steroids secreted by an ovary grafted to the spleen do not reach the peripheral circulation; they are degraded to inactive metabolites in the liver, and cannot exert their systemic feedback inhibition. The increasing gonadotropin secretion stimulates the growth of the transplanted ovary. This effect can be diminished or prevented by systemic application of gonadal steroids. Several studies were reported by: Mardones et al. 1956; Shibley 1962; Biskind and Biskind 1990; D'Albora et al. 1992. The method by Mardones et al. (1956) for the guinea pig and by Desclin (1959) for the rat was modified by H. G. Vogel and S. Jung (unpublished data, 1962).

PROCEDURE

Female Sprague-Dawley rats weighing 100–120 g are anesthetized. The abdominal wall is opened by an incision lateral to the linea alba. Both ovaries are removed. One ovary is freed from the capsule and connective tissue and weighed to the nearest 0.1 mg. An incision is made at the cranial end of the spleen forming a deep pocket. The decapsulated ovary is pushed into this pocket. The incision is closed, and animals are treated with daily subcutaneous injection of an estrogen (e. g., 0.01, 0.1, 1.0, and 10 μ g estradiol in 0.1 ml sesame oil) or a gestagen (e. g., 0.001, 0.01, 0.1, and 1.0 mg 6 α -methyl-17 α -acetoxyprogesterone) for a period of 5 weeks. Controls receive the vehicle only. In controls, the weight of the ovary may increase about

eightfold, from an average weight at implantation of about 10.0 mg to values around 80.0 mg.

Estradiol at doses starting from 0.1 μ g and 6 α -methyl-17 α -acetoxyprogesterone at a dose of 1.0 mg suppress ovarian growth completely by inhibition of LH secretion. The weight of the uterus is increased by direct action of estradiol too.

EVALUATION

Dose–response curves of test compounds and reference standards are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The ovary–spleen transplantation method is similar to the parabiosis experiment, with much less stress to the animals.

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N.7.1.7.5

Inhibition of Fertility

PURPOSE AND RATIONALE

Fertility in rats can be inhibited by treatment of females with steroids that inhibit pituitary gonadotropin secretion or compete at the progesterone receptor. The test was used for contraceptive steroids (Shibley 1962; Philibert et al. 1985; Dhar et al. 1994).

PROCEDURE

A colony of about 100 adult female Sprague-Dawley rats weighing between 200 and 250 g is established. Daily vaginal smears are taken at noon for 5 days. Then 15 regularly cycling females in proestrus are selected and caged separately. The first drug dose is administered at 3:00 p.m. (day 1). At 5:00 p.m. two males are placed with each female. On day 2, vaginal smears are taken for sperm count at 8:00 a.m.. The sec-

ond drug dose is administered at 4:30 p.m. On day 3, vaginal smears are taken for sperm count at 8:00 a.m. again. The third drug dose is administered and sperm counts are taken at 4:30 p.m. The males are then removed. From the 4th to the 7th day the test drugs are administered in the morning. Controls receive the vehicle only. On day 9, the animals are sacrificed and the uterus examined for implantation sites.

EVALUATION

Compounds which prevent conception, as evidenced by the absence of implantation sites at autopsy, were given further consideration. Minimal effective doses preventing ovulation in all animals of a test group were determined.

REFERENCES AND FURTHER READING

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N.7.2

Prolactin

There are considerable sequence homologies in the structures of growth hormone, prolactin and placental lactogen; however, their biological activities are rather different and each hormone exerts its specific pattern of activities on tissues, with growth hormone being an anabolic hormone, and prolactin predominantly a reproductive hormone.

N.7.2.1

General Considerations

The classical procedure for the bioassay of prolactin is based on the work of Riddle et al. (1939); namely, the increase in the weight of the crop sacs of doves and pigeons. Other methods are based on the induction of secretory changes in the mammary glands of rodents. Most of these methods can now be replaced by cell-based assays using suitable cell lines responding to prolactin.

The clinical measurements rely on immunoassays (Shiu and Friesen 1976; Jacobs 1979; Jeffcoate et al.

1986). Due to the species specificity of prolactin, a special radioimmunoassay for rats was developed.

Leroy-Martin et al. (1995) reported an immunocytochemical study of human prolactin receptors using antiidiotypic antibodies in human breast cancer.

REFERENCES AND FURTHER READING

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N.7.2.2

Radioimmunoassay of Rat Prolactin

PURPOSE AND RATIONALE

Prolactin is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin releasing hormone activity or prolactin inhibiting factor activity, a homologous assay is necessary. The reagents for rat prolactin and guidance for the method are provided by National Pituitary Agency, USA (Jeffcoate et al. 1986).

PROCEDURE

Reagents

Standard: NIAMDD-rat-prolactin-RP-1

Antiserum: rabbit-anti-rat-prolactin (NIAMDD-S-6)

Tracer: ¹²⁵I-rat-prolactin (NIAMDD-I-3)

Second antibody: goat-anti-rabbit-gammaglobulin (Cat. No. OTP 14/15)

Buffer: 0.01 M-phosphate-saline/0.1% bovine serum albumin, pH 7.4

Assay Procedure

Standards: 0.03–1 ng/tube, 200 µl/tube

Antiserum: 1:4000 100 µl/tube

Tracer: specific activity 250 $\mu\text{Ci}/\mu\text{g}$, 10,000 cpm in 100 $\mu\text{l}/\text{tube}$

Standards (or sample) are incubated with antiserum for 24 h at +4°C; the tracer is added and incubated for another 48 h. Then the second antibody (1:50), 200 $\mu\text{l}/\text{tube}$, is added, and incubated for 48 h at +4°C. Separation is performed with 1.0 ml ice-cold phosphate-buffered saline, pH 7.4, the vials spun at 1300 g for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma-counter.

Counting equipment required: gamma spectrometer.

EVALUATION

Data processing: standard curves and sample data are calculated by any suitable computer program, e. g., using a spline function.

REFERENCES AND FURTHER READING

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N.7.2.3

Pigeon Crop Method

PURPOSE AND RATIONALE

This is again a historical bioassay no longer applied. Riddle and Bates (1939) showed that the secretion of “crop milk” by pigeons and doves is initiated and maintained by a factor from the anterior pituitary of these birds that is identical to the lactogenic hormone from mammals. Suitable assay methods have been developed on this basis (Meites and Turner 1950; Segaloff 1962; Cowie and Forsyth 1995). Pigeons are exquisitely sensitive to lactogenic hormone, and the assay can be done with considerably less material than the mammalian assays. The most sensitive assay is the so-called micromethod of local intracutaneous injections of the crop sac.

PROCEDURE

Pigeons of either sex (2–3 months old), but of uniform strain, e. g., White Carneaux, are injected intramuscularly (intrapectorally) with various doses of the test preparation or the standard once daily for 4 days. On the 5th day, the birds are sacrificed. A midventral incision is made through the skin and crop wall from keel to head. The contents of the crop and the adhering crop-milk are removed. The two lateral pouches are removed, the fat cleaned from the back of the glands

and the wet weight of the glands determined. Thus, the unstimulated tissue in the dorsal midline and that around the proximal and distal opening of the crop are not weighed.

EVALUATION

Mean values of at least two doses of test preparation and standard are plotted versus logarithms of doses, and the potency ratios with confidence limits calculated.

MODIFICATIONS OF THE METHOD

The micromethod is based on the observation that only a small area directly over the site of injection is stimulated when lactogenic hormone is injected intradermally over the crop sac forming a “bleb” at the site of injection. A direct comparison of the potency of two different preparations can be made by a similar injection over the other crop sac. The birds are sacrificed on the 5th day and the entire crop sacs removed and examined by transmitted light (Lyons and Page 1935).

REFERENCES AND FURTHER READING

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N.7.2.4

Lactation in Rabbits

PURPOSE AND RATIONALE

Only the rabbit and guinea pig have proven satisfactory for mammalian assays of lactogenic hormones. The assays are based on the prolactin-induced mammary growth and milk secretion of pseudopregnant animals.

PROCEDURE

Pseudopregnancy is induced in mature estrus rabbits by intravenous injection of 50 IU of human chorionic gonadotropin. On the 14th day, the rabbits are examined for the presence of well-developed mammary glands characteristic of pregnancy. Various doses of the test preparation or the standard of lactogenic hormone are injected subcutaneously once daily for 6 days. On the 7th day the animals are sacrificed and

the abdominal skin is incised in the midline and separated from the mammary gland underneath. The degree of enlargement of the glands with secretion is rated as follows:

- absence of response
- + all ducts are filled with milk
- ++ all ducts and most lobules are filled with milk though not greatly thickened
- +++ entire gland is filled with milk
- ++++ mammary glands are greatly extended with milk throughout.

EVALUATION

The mean values for groups of six rabbits are compared with the values of the standard groups.

MODIFICATIONS OF THE METHOD

A more sensitive rabbit assay method is based on the ability of lactogenic hormone to act directly on mammary tissue (Lyons 1942). Small amounts of test compound are injected directly into one or more of the six milk ducts of a castrated, rabbit pretreated with estrone-progesterone. A localized lactation appears in the gland sector stimulated. A dose-dependent reaction was found. The assay is suitable for prolactin, placental lactogen and growth hormone.

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N.7.3

Growth hormone (GH)

N.7.3.1

General Considerations

Growth hormone (GH) has been isolated from the pituitary glands of several species including humans and is species specific; for therapy it has been replaced

by rDNA preparations. Several methods have been described for the detection and assay of GH activity (Russell 1955; Hughes 1985; Isaksson 1985; Rudd 1991).

Human GH consists of 190 amino acids with two disulfide bridges between cysteine residues. Human GH synthesis is controlled by two genes: hGH-N and hGH-V.

GH secretion is stimulated by growth-hormone-releasing hormone (see Sect. N.9.5). GH is released in a pulsatile fashion and has a half-life of about 10 min.

Receptors for GH are found in several organs, mainly in liver and muscle.

Receptor binding assays (Ilondo et al. 1991) and radioimmunoassays for GH in several animal species (Greenwood et al. 1963; Peake et al. 1978) and for somatomedins (Chochinov and Daughaday 1978) have been developed.

Amit et al. (1992) measured serum GH by radioimmunoassay and GH-binding protein by a binding assay with dextran-coated charcoal separation.

An immunoradiometric assay for Gh was described by Hofland et al. (1989).

Mertani et al. (1995) studied the cellular localization of the GH receptor/binding protein in the human anterior pituitary gland.

A high-performance receptor binding chromatography assay for GH was described by Roswell et al. (1996).

Strasburger et al. (1996) developed an immunofunctional assay for human GH (hGH). An anti-hGH monoclonal antibody recognizing binding site 2 of hGH is immobilized and used to capture hGH from the serum sample. Biotin-labeled recombinant GH-binding protein in a second incubation step forms a complex with those hGH molecular isoforms that have both binding sites for the receptor. The signal is detected after a short third incubation step with labeled streptavidin.

Functional characterization of monoclonal antibodies specific to GH receptor is described by Wang et al. (1996).

REFERENCES AND FURTHER READING

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- dissolved in saline are injected subcutaneously daily over a period of up to 20 days. During this time, weight gains between 10 and 40 g can be achieved. A straight line relationship exists between the logarithm of the daily dose and the growth response by body weight increase (Marx et al. 1942).

EVALUATION

The weight gains after administration of two doses of the test preparation and of the standard are used for 2+2-point assays, and potency ratios are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The test needs a relatively large amount of test material. In spite of the species specificity of growth hormone, rats respond to growth hormone from many other species over a limited time period, before developing neutralizing antibodies.

MODIFICATIONS OF THE TEST

Immature female rats hypophysectomized at 26–28 days of age can be used (Li et al. 1945; Groesbeck and Parlow 1987). Smaller amounts of test substance are necessary for weight gain than in adult rats.

A cell proliferation assay using a stable clone of the myeloid cell line FDC-P1, transfected with the full-length growth hormone receptor (FDC-P1-hGRH), was described by Roswell et al. (1996) as an alternative to the classical hypophysectomized rat weight gain bioassay.

N.7.3.2

Weight Gain in Female Rats (“Growth Plateau Rats”)

PURPOSE AND RATIONALE

Female rats 6 months of age, having reached maturity, gain weight at a very slow pace; the slowing down of the growth rate is described as “plateauing”. Such rats can readily be induced by growth hormone to accelerate growth and weight gain (Greenspan et al. 1950; Papkoff and Li 1962).

PROCEDURE

Groups of ten adult female rats (Long-Evans or Wistar strain), 6 months old and weighing between 220 and 280 g, are used. Only animals which fail to gain more than 10 g in a 20-day period are used. At least two doses of the hormone preparation and the standard

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say for recombinant human growth hormone. *Biologicals* 24:25–39

N.7.3.3

Tibia Test in Hypophysectomized Rats

PURPOSE AND RATIONALE

Hypophysectomy is followed by cessation of epiphyseal growth due to growth hormone deficiency. The width of the epiphyseal cartilage is markedly reduced after hypophysectomy. Administration of growth hormone to hypophysectomized rats induces a remarkable increase in the width of the epiphyseal cartilage plate.

PROCEDURE

Female rats of the Long-Evans or Sprague-Dawley strains are hypophysectomized at the age of 26–28 days. They are used for the bioassay 12–14 days after the operation. The increase of body weight during this period of time has to be less than 0.5 g per day to indicate complete hypophysectomy. Six to ten animals are used for each group of two doses of test preparation and standard. The solutions are administered intraperitoneally twice daily for 4 days.

On the 5th day, the animals are sacrificed, both tibiae are dissected free of soft tissue, and the bones split in half with a sharp razor at the proximal end in the mid-sagittal plane. The halves are washed in water for 10 min, immersed in acetone for 6 min, and washed again in water for 3 min. They are then placed in 2% silver nitrate solution for 2 min and rinsed with water. During the water rinse, they are exposed to a strong light which turns the calcified portions of the bone dark brown. The stained tibiae are transferred to a microscopic stage and the width of the uncalcified cartilage plate, which does not stain and remains white, is measured under low power with a calibrated micrometer eyepiece. Ten individual readings are made across the epiphysis.

EVALUATION

Mean values are obtained from a total of 20 readings for each bone specimen. They are averaged for each dose group. With a 2+2-point assay, the potency ratio with confidence limits versus the standard is calculated.

CRITICAL ASSESSMENT OF THE METHOD

The test has been firmly established as a standard method for assessing growth-promoting activity. For

epiphyseal measurement, morphometry may be applied.

MODIFICATIONS OF THE METHOD

Bentham et al. (1993) described a double-staining technique for the detection of growth hormone and insulin-like growth factor-1 binding to rat tibial epiphyseal chondrocytes that were incubated with biotinylated ligands with or without an excess of unlabeled ligands, followed by incubation with Vectastain ABC complex, which was then reacted with diaminobenzidine. Double staining was accomplished by carrying out the first reaction with diaminobenzidine in the presence of nickel ammonium sulfate to give a black precipitate, followed by incubation with the second ligand, then the ABC complex and finally diaminobenzidine in the absence of nickel ammonium sulfate to give a brown stain.

REFERENCES AND FURTHER READING

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N.7.3.4

³⁵S Uptake

PURPOSE AND RATIONALE

Cartilage contains glucosaminoglycans, e.g., chondroitin sulfate. The uptake of labeled sulfate into cartilage is greatly reduced after hypophysectomy and restored after growth hormone application. This phenomenon can be used as the basis of a bioassay of growth hormone activity (Collins and Baker 1960; Papkoff and Li 1962).

PROCEDURE

Female Sprague-Dawley rats are hypophysectomized at 21 days of age and used for experimentation 3 weeks later. The animals are given intraperitoneal injections of growth hormone together with radioactively labeled sulfate once daily for 4 days. Eight to ten animals are

used for at least two doses of test preparations (growth hormone derivatives) and standard. The rats are sacrificed 24 h after the last injection and the amount of radiolabeled sulfate present in the seventh rib cartilage is determined. A linear relationship exists between the uptake of radiolabeled sulfate and the hormone given over a range of 3–20 µg per day for 4 days.

EVALUATION

Mean values for each group and the potency of the test preparation versus standard calculated with confidence limits are calculated in a 2+2-point assay.

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N.7.3.5

Inhibition of Glucose Uptake in Adipocytes in Vitro

PURPOSE AND RATIONALE

The conversion of glucose to lipid in murine adipocytes is dose-dependently inhibited by human growth hormone (hGH). A sensitive *in vitro* bioassay was developed by Foster et al. (1993).

PROCEDURE

3T3-F442A embryonic murine fibroblasts (preadipocytes) are grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.1 µg/ml fungizone. The cells are plated in 60- or 100-mm plastic culture dishes at a density of 200 cells/cm² and grown to confluence at 37°C under a humidified atmosphere of 90% air/10% CO₂ in medium supplemented with 25 mM glucose and 10% calf serum. Medium is replaced every 2–3 days. Once confluent, the fibroblasts are converted to cells with the characteristics of adipocytes by incubation for 48 h in medium supplemented with 25 mM glucose, 10% fetal bovine serum, 0.5 mM methylisobutylxanthine, 2 µg/ml insulin, and 250 nM dexamethasone. This medium is then replaced with medium containing 10% fetal bovine serum and 2 µg/ml insulin with changes made every 2 days for 5–8 days until at least 70% of the cells have the characteristics of adipocytes as assessed by phase contrast microscopy.

For bioassays, the medium consists of DMEM containing 5.5 mM glucose, 2% BSA, 25 nM dexametha-

sone, 37 nM estradiol, 10 µg/l insulin, and 0.1 µCi/ml uniformly labeled [¹⁴C]D-glucose. Cultures are co-incubated with increasing concentrations of 22-kDa human growth hormone (0.313–40 µg/l) as standard or test substance or medium (controls) for 24 h. For determination of hGH in patients, 100 µl or 200 µl serum is added. After incubation, the medium is removed and discarded. The cells are treated with Doles reagent (one part heptane, four parts isopropanol, 0.1 part 1 N H₂SO₄), the plates scraped, and the contents transferred to a glass tube. Lipids are extracted by the method of Dole and Meinertz (1969), and radioactivity of the lipid is determined by scintillation counting in a liquid scintillation spectrophotometer. Results are expressed as ¹⁴C counts per minute per dish. Lipid accumulation in controls without hGH is taken as 100%. Logarithmic doses of 0.313–40 µg/l hGH result in a linear decrease of lipid accumulation. The assay is rather specific for 22-kDa hGH.

EVALUATION

From dose–response curves, activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Xu et al. (1995) studied the effects of GH antagonists on 3T3-F422A preadipocyte differentiation. The antagonists not only failed to induce adipose differentiation, including late marker gene expression (adipocyte protein 2), immediate early gene expression (c-fos), and tyrosine phosphorylation of intracellular proteins, but also antagonized GH-induced c-fos expression and phosphorylation of proteins of apparent molecular mass 95 kDa.

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N.7.3.6

Eluted Stain Bioassay for Human Growth Hormone

PURPOSE AND RATIONALE

Ealey et al. (1988, 1995) and Dattani et al. (1993, 1995) developed an eluted stain bioassay

(ESTA) for human growth hormone (hGH). This assay is based on the production of MTT–formazan [MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium] by quiescent Nb2 cells under the influence of the hormone.

PROCEDURE

Rat Nb2 lymphoma cells are grown in suspension culture which consists of RPMI medium (Gibco) containing 50 U penicillin/ml, 50 µg streptomycin/ml, 2×10^{-3} M L-glutamine, 10% fetal calf serum and 10% horse serum. The cells are incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C.

Prior to the bioassay of hGH, the cells are transferred to a quiescent medium, which is identical in composition to the growth medium described above except that the FCS is reduced from 10% to 1%. This slows down the rate of cell division and reduces the optical density of the unstimulated control in the subsequent ESTA bioassays by about 50%. Incubation is continued in a humidified atmosphere of 5% CO₂/95% air at 37°C for 24 h.

For the bioassay, the cells are transferred to the bioassay medium, which is the same as the growth medium but without FCS. The cells are plated out into 96-well microtiter plates, such that a final density of 2×10^5 cells/ml is obtained. Usually 50 µl of cell suspension at 4×10^4 cells/ml is added to each well. This is followed by the addition of 50 µl of various concentrations of test compound or standard (0.1–10 mU GH/l). The cells are incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C for 96 h.

At the end of the bioassay incubation, the colorimetric endpoint is determined by the addition of 10 µl of MTT bromide solution (5 mg/ml in phosphate-buffered saline containing 0.1 mg/ml CaCl₂ and MgCl₂ · 6H₂O, pH 7.3) to each well: incubation is continued for 40 min at 37°C in a dry incubator. During this time, activated cells reduce the yellow MTT salt to its purple formazan. After the 40-min incubation, 50 µl of 10% Triton X-100 in 0.1 M HCl is added to each well and the plate gently shaken for 30 min at room temperature. Bioassay responses of the 96 wells are quantified with a Biorad microtiter plate reader at optical densities at a test wavelength of 595 nm and a reference wavelength of 655 nm to correct for differential scattering.

EVALUATION

The determinations for all experiments are made from triplicate or quadruplicate microcultures and the results expressed as means and standard deviations.

Dose–response curves for standard and test compounds are established.

MODIFICATIONS OF THE METHOD

The sensitivity of the assay can be increased by addition of ionic zinc (Dattani et al. 1993, 1995; Strasburger and Dattani 1997).

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N.7.3.7

Reverse Hemolytic Plaque Assay for Growth Hormone

PURPOSE AND RATIONALE

The reverse hemolytic plaque assay as described by Neill and Frawley (1983), Luque et al. (1986), and Smith et al. (1986) can be used to determine growth hormone (GH) secretion by dispersed rat pituitary cells (Niimi 1994a, 1994b).

PROCEDURE

Anterior pituitary lobes of male Sprague-Dawley rats weighing 200–250 g are collected and minced into approximately 1-mm³ fragments. The pituitary cells are dispersed and transferred to a siliconized flask containing 3 mg/ml trypsin in Dulbecco's phosphate-buffered saline containing 0.1% BSA and antibiotics, and incubated in a water bath for 5 min at 37°C. After gentle trituration by a siliconized pipette, the cells are separated by centrifugation and rinsed with phosphate-buffered saline containing 2 mg/ml DNase (type I; Sigma, St. Louis, Mo., USA). The dispersed cells are separated by centrifugation and rinsed once in phosphate-buffered saline with 1.5 mg/ml trypsin inhibitor (type II-L, Sigma). The cells are washed 5 times with Dulbecco's modified Eagle's medium (DMEM) containing 0.1% BSA and resuspended. Ovine erythrocytes are coupled with staphylococcal protein A

(Sigma) in the presence of 0.9% chromium chloride in normal saline. Pituitary cells (10^6 cells/ml) are combined with an equal volume of a 30% solution of protein-A-coupled erythrocytes in DMEM containing 0.1% BSA and antibiotics. The cell mixture is infused into a poly-L-lysine coated Cunnigham slide chamber and preincubated at 37°C, 95% air/5% CO₂, for 50 min. After preincubation, the chamber is rinsed in DMEM-BSA and placed in Petri dishes containing DMEM and 10% horse serum containing 1% nonessential amino acids before performing the reverse hemolytic plaque assay.

After 24 h of co-incubation, the chambers are rinsed with DMEM-BSA. Monkey anti-rat GH serum diluted 1:150 in assay medium is then infused into the chambers alone or with different secretagogues and incubated for 2 h. Plaque development is initiated by infusion of guinea pig complement (Gibco) at a final dilution of 1:40. The reaction is terminated after 0.5 h by the infusion of B-5 fixative (6 g HgCl₂ and 1.25 g sodium acetate in 90 ml distilled water, add 10 ml 37% formaldehyde immediately before use). The pituitary cells are stained with 0.5% toluidine blue to facilitate observation of the hemolytic plaques.

EVALUATION

In each experiment, each concentration of secretagogue or vehicle is run in duplicate, and 150–200 cells/slide are counted. Two separate experiments have to be performed. The plaque area is measured by using a calibrated ocular reticule. The area of 50 plaques per slide is measured. Statistical analysis is performed by Student's *t*-test and one-way analysis of variance.

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N.7.3.8

Determination of Growth Hormone Isoforms by 22-kDa GH Exclusion Assay

PURPOSE AND RATIONALE

Human growth hormone exists in variety of isoforms. In the pituitary, the most abundant isoform is 22-kDa GH, while other isoforms (non-22-kDa GH) are present in variable amounts. Boguszewski et al. (1996); Strasburger and Dattani (1997) described an analytical approach that focuses on the isoforms differing from monomeric and oligomeric 22-kDa GH.

PROCEDURE

Reagents

- Recombinant 22-kDa human GH (Genotropin, Pharmacia, Uppsala, Sweden)
- 22-kDa GH-specific monoclonal antibody (MCB), (Genentech, San Francisco, Calif., USA)
- magnetic polystyrene beads coated with rat anti-mouse IgG 1 (Dynabeads M-450), (Dyna, Oslo, Norway)
- polyclonal antibody-based IRMA (Pharmacia, Uppsala, Sweden)
- assay buffer containing phosphate-buffered saline, 5 g/l BSA, 5 ml/l Tween-20, 0.1 g/l thiomersol.

Assay Procedure

A 100- μ l aliquot of serum or test solution is mixed with either 10 μ l of assay buffer containing MCB (final concentration 0.3 μ mol/l) or 10 μ l of assay buffer without MCB. The samples are incubated for 24 h at room temperature. A 160- μ l aliquot of magnetic beads coated with rat anti-mouse IgG (concentration 4×10^5 beads/ μ l) is added to the samples. After further incubation for 2 h at room temperature with gentle agitation in a rotator, the tubes are put in contact with a magnetic device, Dynal MPC-E (Dynal, Oslo, Norway), for 1 min. The magnetic beads with the 22-kDa GH-MCB complexes are attracted by the magnet. While the tubes are in the magnetic device, 50- μ l aliquots of the supernatant are transferred to new tubes for measurement of non-22-kDa GH levels in duplicate by the polyclonal antibody-based IRMA. The same procedure is performed for samples incubated with assay buffer (without addition of MCB) to determine total GH concentration.

EVALUATION

The amount of non-22-kDa GH isoforms is expressed as a percentage of total GH concentration. The Mann-

Whitney *U* test (two-tailed) is used to compare the percentage of non-22-kDa GH isoforms between the groups. The method is an example of the increasing use of physicochemical methods to characterize the heterogeneity of hormone preparations obtained by rDNA methods.

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N.7.3.9

Steroid Regulation of Growth Hormone Receptor and GH-Binding Protein

PURPOSE AND RATIONALE

Gabrielsson et al. (1995) studied steroid regulation of growth hormone receptor (GHR) and GH-binding protein (GHPB) mRNAs in the rat. GHR and GHPB, arising from alternative splicing of the same gene, show a sexually dimorphic and GH-dependent expression pattern. Multiple alternative 5'-untranslated regions are present in GHR and GHPB transcripts in the rat, one of which, GHR₁, has been shown to be liver specific and found at higher levels in females.

PROCEDURE

Animal Treatment

For human GH treatment, groups of Wistar rats are implanted subcutaneously with osmotic minipumps delivering recombinant hGH at 200 µg/day for 7, 12, or 14 days. Steroid-treated animals are implanted subcutaneously with slow-release pellets delivering estradiol (E₂, 25 ng/day), testosterone propionate (12.5 µg/day) or corticosterone (3.5 µg/day) for up to 2 weeks. At the end of each study, animals are sacrificed and samples of blood and liver rapidly removed.

Protein Binding (GHPB) Assay

Plasma GHPB levels are measured by radioimmunoassay (RIA). Recombinant rat GHPB is used for iodination and reference preparation (Carmignac et al. 1992).

GH-Receptor-Based Assays

Liver samples are homogenized in 0.3 M sucrose containing 3 mM imidazole HCl, pH 7.4 (Carmignac et al. 1993). Samples of 100 µl (~2 mg protein) are incu-

bated in duplicate at 22°C with radio-iodinated bovine GH (100 µl, 20,000 cpm) and 100 µl buffer (25 mM Tris-HCl, pH 7.4, containing 10 mM CaCl₂ and 0.1% BSA). After 2 h, 3 ml cold (4°C) buffer is added, the tubes centrifuged at 2700 g for 30 min, and the radioactivity in the pellets determined. Nonspecific binding is estimated in the presence of 1 µg unlabeled bovine GH and is subtracted from total binding to derive percentage specific bovine GH binding per milligram protein.

Generation of RNA Probes

Using an *in vitro* transcription kit, probes are labeled with [³⁵S]uridine triphosphate for solution hybridization assay and [³²P]uridine triphosphate for Northern blots and RNase protection assays. Total GHR coding region transcripts are measured using an antisense probe corresponding to a 423-nucleotide (nt) *NcoI-KpnI* fragment (nt 989–1411) of the λ1 clone of the rGHR inserted into the pT7T318U vector. This sequence spans the transmembrane domain and part of the intracellular domain of the GHR and is not present in GHPB mRNA. Total GHPB transcripts are determined using a 46-nt oligonucleotide probe complementary to the alternate splice sequence encoding the GHPB hydrophilic tail. This probe does not detect GHR transcripts. GHR₁-containing transcripts are measured using a probe complementary to this 5'-UTR alternative exon sequence.

Northern Blots

Total RNA is prepared using the method of Chomczynski and Saachi (1987). RNA is run overnight on a 2.2 M formaldehyde/3-*N*-morpholine propanesulfonic acid agarose gel, transferred to a Nylon membrane and cross-linked using a Stratilinker (Stratagene, La Jolla, Calif., USA). Membranes are prehybridized for 2–4 h at 60–65°C in 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 5 × Denhardt's solution, 50 mM Na₂HPO₄, 0.2% sodium dodecyl sulfate (SDS), 250 µg/ml salmon sperm DNA, and 100 µg transfer RNA/ml, and incubated with ³²P-labeled probe [(1–3) × 10⁶ cpm/ml] at the same temperature in 50% formaldehyde, 5 × SSC, 1 × Denhardt's solution, 20 mM Na₂HPO₄, 0.2% SDS, 10% dextran, 100 µg/ml salmon sperm DNA, and 100 µg tRNA/ml. After washing (60°C and 20 min/wash, starting at 3 × SSC/0.1% SDS and reducing to 0.3 × SSC/0.1% SDS) membranes are exposed to X-ray film with intensifying screens at –80°C or are detected by phosphorimaging.

Solution Hybridization Assays

Hepatic mRNAs are quantified by solution hybridization after the method of Möller et al. (1991). Liver is homogenized in 10 mM Tris-HCl, pH 7.5, containing 1% SDS and 5 mM EDTA, after proteinase K digestion. Total nucleic acids (TNAs) are extracted with phenol/chloroform, precipitated with ethanol, and the pellets dissolved in 0.2×10 mM Tris-HCl, pH 7.5, containing 1% SDS and 5 mM EDTA. For each assay, 10–100 μ g TNAs is hybridized in duplicate with 35 S-labeled RNA probes (20,000–30,000 cpm) at 50°C overnight in 40 μ l, 21 mM Tris-HCl buffer, pH 7.5, containing 600 mM NaCl, 4.5 mM EDTA, 7.5 mM dithiothreitol, 0.1% SDS, and 25% formamide. Standard tubes contain known quantities of target RNA, transcribed from the sense strand of the appropriate plasmid and quantified by absorption at 260 nm.

After digestion with RNase A (40 μ g) and RNase T₁ (2 μ g) protected fragments are precipitated with 10% trichloroacetic acid, are collected by filtration (GF/C paper, Whatman), detected by scintillation counting, and expressed as specific mRNA levels (attomoles per μ g TNA).

RNase Protection Assay

Total RNA (20 μ g) is hybridized under the same conditions as above, except that a 32 P-labeled probe (500,000 cpm) is used. After overnight incubation at 50°C, the samples are treated with 300 μ l of a solution of 10 mM Tris, pH 7.5, 5 mM EDTA, and 300 mM NaCl, containing RNase A (40 μ g/ml) and RNase T₁ (2 μ g/ml), for 30 min at room temperature. After incubation with 50 μ g proteinase K and 20 μ l 10% SDS for 15 min at 37°C, samples are extracted with phenol/chloroform and ethanol precipitated with 10 μ g tRNA as carrier. The pellets are dissolved in gel-loading buffer (10 mM EDTA and 1 mg/ml bromophenol blue in 80% formamide), denatured at 85°C for 4 min, and run on an 8 M urea/6% polyacrylamide gel. Detection is by autoradiography or phosphorimaging.

EVALUATION

Data are calculated as mean \pm SEM. Differences between treatment groups are assessed using Student's *t*-test, or analysis of variance followed by Student-Newman-Keul's or Dunnett's tests.

MODIFICATIONS OF THE METHOD

Nilsson et al. (1995) described the expression of functional growth hormone receptors in cultured human osteoblast-like cells.

To measure the absolute number of mRNA molecules encoding the growth hormone receptor in human tissue, Martini et al. (1995) developed a quantitative polymerase chain reaction assay.

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N.7.4**Adrenocorticotropin (ACTH)****N.7.4.1****Adrenal Ascorbic Acid Depletion****PURPOSE AND RATIONALE**

This is now a historical assay, which however has been used extensively for standardization of ACTH preparations. The administration of pituitary adrenocorticotropin hormone (ACTH) is followed by a decrease in the amount of ascorbic acid present in the adrenals. The depletion of adrenal ascorbic acid is a function of the dose of ACTH administered. This relationship was used for a quantitative assay of ACTH by Sayers et al. (1948). The method has been selected for standardization of ACTH by several Pharmacopoeias, e. g. The United States Pharmacopeia USP 23 (1995), Deutsches Arzneibuch (1986) and British Pharmacopoeia (1988). Furthermore, the test has been used for evaluation of synthetic corticotropin analogs (Geiger et al. 1964; Vogel 1965, 1969a, 1969b). A similar test is used for luteinizing hormone action in the rat ovary.

PROCEDURE

Male Wistar rats weighing between 100 and 200 g are hypophysectomized 1 day prior to the test. The range of weights in any one test should not exceed 15 g. For one test with three doses of test preparation and standard, at least 36, preferably 60, hypophysectomized rats are necessary.

Solutions

Five units of the International Standard for corticotropin (Bangham et al. 1962) or an amount of test preparation supposed to contain about 5 units are dissolved in 0.25 ml of 0.5% phenol solution and diluted with 8.1 ml of 15% gelatin solution. In this way, 0.5 ml contains 300 mU ACTH. Then 3 ml of this solution is diluted with 6.0 ml gelatin solution (to prevent adsorption to glassware), resulting in 100 mU ACTH per 0.5 ml. Then 3 ml of this solution is again diluted with 6.0 ml gelatin solution, resulting in a content of 33 mU ACTH per 0.5 ml.

The hypophysectomized rats are randomly distributed to six groups. Each rat receives subcutaneously 0.5 ml of one of the various concentrations of test preparation or standard. Three hours after injection, the animals are anesthetized, both adrenals removed, freed from extraneous tissue and weighed. The rats are sacrificed and the skull opened to verify completeness of hypophysectomy.

The adrenals are homogenized in 4% trichloroacetic acid and the ascorbic acid determined according to the method of Roe and Kuether (1943). Other methods have been described.

ASCORBIC ACID DETERMINATION**Reagents**

First 100 mg L-ascorbic acid is dissolved in 100 ml 4% trichloroacetic acid and 20 ml of this solution is diluted with 4% trichloroacetic acid to achieve a 0.2% ascorbic acid solution; 2 ml of this solution is diluted with 4% trichloroacetic acid to achieve a 0.02% ascorbic acid solution. Sulfuric acid (85%) is obtained by adding 900 ml concentrated sulfuric acid to 100 ml distilled water. Then 2 g dinitrophenylhydrazine is dissolved in 100 ml of 9 N H₂SO₄ (75 ml distilled water and 25 ml concentrated sulfuric acid), and 6 g thiourea is dissolved in 100 ml distilled water.

Calibration

Trichloroacetic acid (4%) is added to 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 ml of the 0.02% ascorbic acid solution and 1.0, 1.5, and 2.0 ml of the 0.2% ascorbic acid solution to reach a final volume of 8.0 ml. Then

100 mg charcoal is added to each sample and thoroughly mixed by shaking for 1 min. After 5 min the solutions are filtered. An aliquot of 0.1 ml of the 6% thiourea solution is added to 2.0 ml of the filtrate followed by 0.5 ml dinitrophenylhydrazine solution. The mixture is shaken and heated for 45 min at 57°C in a water bath. The solutions are placed in an ice-cold water bath and with further cooling 2.5 ml of the 85% sulfuric acid is added. The calibration curve is established at a wavelength of 540 μm using the solutions without ascorbic acid as blank.

Preparation of the Adrenals

Both adrenals are homogenized in glass tubes containing 200 mg purified sand and 8.0 ml of 4% trichloroacetic acid. The reagents are added as described for the calibration curve.

EVALUATION

The potency ratio including confidence limits is calculated with the 3+3-point assay.

MODIFICATIONS OF THE METHOD

The original method, as described by Sayers et al. (1948), used intravenous administration of ACTH and the difference of ascorbic acid in the left adrenal before injection and the right adrenal 1 h after injection as endpoint. Different values of the activity of synthetic peptides versus the International Standard resulted from different ways of administration (Vogel 1965).

The ascorbic acid depletion test can also be performed in dexamethasone-blocked rats. However, different potency ratios of synthetic corticotropin analogs have been found than in hypophysectomized rats (Vogel 1969a). The difference most likely depends on the dexamethasone blocking dose.

Other authors, including British Pharmacopoeia (1988) and Deutsches Arzneibuch (1986), use the 2,6-dichlorophenol-indophenol method for determination of ascorbic acid.

The glands are homogenized in 2.5% metaphosphoric acid with the addition of a small quantity of washed sand. With additional 2.5% metaphosphoric acid a final volume of 10 ml is reached. Then 5 ml of the filtrate is added to 5 ml indophenol acetate solution, and the absorbance of the mixture is read immediately in a photometer with a 520 μm filter. The indophenol acetate solution is prepared by dissolving 15 mg of 2,6-dichlorophenol-indophenol in 500 ml distilled water and dissolving 22.65 g sodium acetate·3H₂O in 500 ml distilled water and mixing equal volumes.

A cytochemical bioassay of corticotropin was described by Chayen et al. (1976).

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sone-blocked rats. The test can be used to measure time–response curves of corticotropin analogs or depot preparations (Vogel 1969a, 1969b). The sensitivity can be increased by determining corticosterone in adrenal venous blood after cannulation of the adrenal vein (Retiene et al. 1962).

PROCEDURE

Male Sprague-Dawley rats weighing 150–200 are injected subcutaneously 24 h and 1 h prior to subcutaneous injection of the ACTH preparation or the standard with 5 mg/kg dexamethasone in oily solution. Eight rats are used for each dose of test preparation or standard. At increasing time intervals after ACTH injection, the rats are anesthetized with 60 mg/kg pentobarbital i.p. and blood is withdrawn by venipuncture (alternatively by retro-orbital puncture). Next, 1 ml plasma is diluted with 2 ml distilled water and extracted (washed) with 5 ml petrol ether to remove the lipids. The petrol ether is discarded and 2 ml of the water layer is extracted twice with 5 ml methylene chloride by vigorous shaking for 15 min. The methylene chloride phase is separated by centrifugation. Both methylene chloride extracts are unified and shaken with 1 ml ice-cold 0.1 N NaOH. The water phase is immediately removed and the methylene chloride extracts dried by addition of dry sodium sulfate. A 5-ml aliquot of the methylene chloride extract is mixed with 5 ml of the fluorescence reagent (7 parts concentrated sulfuric acid, 3 parts 96% ethanol, v/v). After vigorous shaking, the methylene chloride phase is removed and fluorescence is measured with primary filters of 436 μm and secondary filters of 530–545 μm . For calibration, concentrations of 0, 20, 50, 100, and 250 $\mu\text{g/ml}$ corticosterone are treated identically and measured in each assay.

EVALUATION

Using three doses of test compound and standard, potency ratios with confidence limits can be determined for each time interval with the 3+3-point assay giving evidence for the duration of action (Vogel 1969a, 1969b).

MODIFICATIONS OF THE METHOD

Pekkarinen (1965) used fluorometric corticosteroid determinations in guinea pigs resulting in highly deviating activity ratios of synthetic and commercial corticotrophins as compared with the international working standard of ACTH.

The contemporary method for the determination of the corticosterone concentration in serum is radioim-

N.7.4.2

Corticosterone Blood Levels in Dexamethasone-Blocked Rats

PURPOSE AND RATIONALE

Corticotropin activity can be measured by the increase of corticosterone in venous blood of hypophysectomized rats (no longer required) or dexametha-

muoassay, requiring much smaller blood samples and reducing the stress to the animals very markedly.

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N.7.4.3

In Vitro Corticosteroid Release

PURPOSE AND RATIONALE

An *in vitro* assay of corticotropin was described by Saffran and Schally (1955). This test has been modified by Van der Vies (1957) and subsequently used by several authors (Stahelin et al. 1965; Vogel 1969a, 1969b).

PROCEDURE

Solutions

“Double Ringer” solution is prepared as follows:

| | | |
|--------|------------------------------|----------|
| 4.50% | (w/v) sodium chloride | 200.0 ml |
| 5.75% | potassium chloride | 8.0 ml |
| 6.10% | calcium chloride | 6.0 ml |
| 10.55% | monopotassium phosphate | 2.0 ml |
| 19.10% | magnesium sulfate | 2.0 ml |
| | double distilled water up to | 545.0 ml |

Final solution:

| | |
|---------|--------------------------------------------------------------------------------------------------------|
| 54.0 ml | double Ringer |
| 26.0 ml | 1% glucose solution (freshly prepared) |
| 29.0 ml | double distilled water |
| 21.0 ml | 1.3% NaHCO ₃ solution which has been gassed with carbon dioxide at room temperature for 1 h |

The final solution is gassed with a mixture of 95% O₂ and 5% CO₂ for 10 min.

Preparation of Adrenals

Male Sprague-Dawley rats ($n=20$) weighing 150–200 g are anesthetized with 50 mg/kg pentobarbital sodium i.p. applying as little handling stress as possible. The adrenals are removed and freed of connective tissue, taking care that the adrenals are not damaged. Each adrenal is carefully cut into four quarters with fine scissors. The eight quarters from each rat are randomly distributed to 20 preweighed incubation vessels filled with 1.5 ml of final solution. The flasks are mounted on Warburg manometers or placed into a suitable shaking water bath and are gassed under continuous shaking for 1 h with a mixture of 95% O₂ and 5% CO₂ at 38°C (= preincubation period).

The flasks are removed from the bath at the end of the preincubation period. The medium is aspirated as much as possible by means of a small tube attached via a collection bottle to the vacuum line. A 1.4-ml aliquot of fresh medium is added to each flask.

To five vessels each of the following solutions are added:

- 10.0 mU/0.1 ml ACTH standard
- 50.0 mU/0.1 ml ACTH standard
- 10.0 mU/0.1 ml test preparation
- 50.0 mU/0.1 ml test preparation
- 50.0 mU/0.1 ml medium (control)

The vessels are again incubated and gassed with a mixture of 95% O₂ and 5% CO₂ at 38°C under continuous shaking for 2 h. Then 1-ml aliquots of the medium in each vessel are transferred to carefully cleaned glass-stoppered tubes containing 2 ml methylene chloride. The tubes are vigorously shaken for 1 min and centrifuged for 5 min. The methylene chloride phase is transferred with a long needle and a syringe to a quartz micro-cuvette and readings are taken at 225, 240, and 255 μ m.

Dry weight of the adrenals is determined by heating the incubation vessels to 150°C for 2 h.

EVALUATION

Extinction values are calculated for the maximum of absorption (Allen 1950) according to the formula:

$$E = E_{240} - \frac{E_{225} + E_{255}}{2}$$

The potency ratios are calculated with the 2+2-point assay.

MODIFICATIONS OF THE METHOD

Saffran et al. (1971) described a flow-through system for the study of adrenocortical function by rat tissue *in vitro*, in which the fluorometric measure of corticosterone is completely automated.

Corticosterone is now conveniently determined by RIA or by a high-performance liquid chromatography (HPLC) method.

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N.7.4.4**Thymus Involution****PURPOSE AND RATIONALE**

This is a classical indirect bioassay based on the steroid response of the target organ and its effect. Administration of corticotropin decreases thymus weight (due to inducing corticosterone secretion), followed by an increase of adrenal weight (Hayashida and Li 1952; Thing 1953; Thompson and Fisher 1953; Fischer 1962). Young rats respond with an involution of the thymus gland to graded doses of corticotropin (Rerup 1958). A similar assay is used for adrenal steroids.

PROCEDURE

Sprague-Dawley or Wistar rats of either sex, 7–10 days of age, weighing 10–15 g are used. Littermates are preferred. The animals are distributed at random to three groups of the standard and three to five groups of the unknown sample. The ACTH standard or test compounds are injected at different doses once daily subcutaneously for 3 days. Twenty-four hours after the last injection, the animals are sacrificed, the thymus dissected out and weighed to the nearest 0.1 mg. The response is expressed as the average of the individual values for each dose level.

EVALUATION

Dose–response curves are established and potency ratios calculated using a 3+3-point assay.

MODIFICATIONS OF THE METHOD

The numerical sensitivity of the assay can be increased using the quotient between the increase of the weight of the adrenals and the decrease of the weight of the thymus gland (Hohlweg et al. 1960) (ratio adrenal weight increase/thymus weight decrease).

Male Wistar rats at an age of about 3 weeks weighing 18–22 g are injected three times daily at 4-h intervals over 3 days with the test preparation or the standard dissolved in 10% gelatin solution. The animals are sacrificed 18 h after the last injection and the adrenals and the thymus gland removed and weighed.

The ratio of adrenal weight to thymus weight provides a steep dose–response curve.

REFERENCES AND FURTHER READING

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N.7.4.5

Receptor Binding Assay for ACTH

PURPOSE AND RATIONALE

As for other hormones, receptors have been used as the test preparations for comparison of the binding affinities of adrenocorticotropin peptides using a cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line (Kapas et al. 1996). Such assays measure the initial membrane binding but not strictly the biological activation, e. g., of adrenal cells or melanocytes.

PROCEDURE

HeLa cells are seeded into 12-well culture plates at a density of 10^6 cells/well. On the 2nd day of culture, the cells are washed as follows: 2 × in 1 ml of ice-cold 0.9% NaCl, 1 × in 1 ml of ice-cold glycine (50 mM glycine, 100 mM NaCl, pH 3.0) for 5 min, 2 × in 0.5 ml of ice-cold 0.9% NaCl. Cells are then incubated for 60 min at 20°C with increasing concentrations of nonradioactive ACTH or various ACTH analogs and the reactions initiated on the addition of [125 I-iodotyrosyl] 23 ACTH[1-39] (2000 Ci/mmol; final concentration 0.1 pmol/l) in DMEM. At the end of the incubation, the medium is removed and the cells washed three times with 0.9% NaCl and then dissolved in 0.5 M NaOH/0.4% sodium deoxycholate. Each point is determined in triplicate, and the radioactivity is measured using a gamma-counter. Specific binding is determined by subtracting from the total binding the radioactivity associated with cells in the presence of 10^{-5} M nonradioactive ACTH.

EVALUATION

Binding parameters are determined using a computer-assisted calculation, e. g., the LIGAND program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

Penhoat et al. (1993) reported the identification and characterization of corticotropin receptors in bovine and human adrenals by covalent cross-linking of radiolabeled ACTH with the bifunctional cross-linking agent disuccinimidyl suberate to cultured bovine adrenal fasciculata reticular cells and to crude plasma membrane fractions prepared from both human and bovine adrenals.

Lebrethon et al. (1994) and Penhoat et al. (1995) studied the regulation of ACTH receptor mRNA and binding sites by ACTH and angiotensin II in cultured human and bovine adrenal fasciculata cells.

Picard-Hagen et al. (1997) found that glucocorticoids enhance corticotropin receptor mRNA levels in ovine adrenocortical cells.

Zavyalov et al. (1995) described the receptor binding properties of peptides corresponding to the ACTH-like sequence of human pro-interleukin-1 α .

Naville et al. (1996, 1997) developed a stable expression model in order to characterize the human ACTH receptor by binding studies and functional coupling to adenylate cyclase.

Schioth et al. (1996) described the pharmacological distinction of the ACTH receptor from other melanocortin receptors in the mouse adrenocortical cell line Y1.

Moreover, melanocortin receptors do not have a binding epitope for ACTH beyond the sequence of alpha-melanocyte-stimulating hormone (Schioth et al. 1997).

CRITICAL ASSESSMENT OF THE METHOD

Binding affinities are determined for structure–activity studies, and the results need to be assessed and confirmed for biological relevance using an assay for the biological response of tissues *in vitro*, and/or the biological effects in animals. These methods are also useful for assessing the sensitivity of hormone-responsive tissues from animals previously treated (adaptive response).

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N.7.5

Thyrotropin (TSH)

N.7.5.1

General Considerations

The thyroid-stimulating hormone (TSH) can be determined by bioassays (effects on the thyroid gland and secretion of thyroid hormones) and its concentration can be measured by immunoassays, as with other polypeptide hormones (Utiger 1979; Meinhold et al. 1994; Spencer 1994). TSH receptors have been identified and their functional regulation has been described (Vassart and Dumont 1992).

Large-scale synthesis of recombinant human thyrotropin has been reported (Cole et al. 1993; Hussain et al. 1996). The role of the thyrotropin receptor has been reviewed by Vassart and Dumont (1992). Castagiola et al. (1992) described a binding assay for thyrotropin receptor autoantibodies using the recombinant

N.7.5.2

Thyroid Histology

PURPOSE AND RATIONALE

Hypophysectomy results in atrophy of the thyroid gland, which is reversed by administration of thyrotropin. In the thyroid of normal young guinea pigs (Junkmann and Schoeller 1932; McGinty and McCullough 1936) or chicks (Jones 1939) characteristic histological changes are observed after administration of thyrotropin, associated with an increase of thyroid weight (goitrogenic response). In classical bioassays, these findings were the basis for standardizing by biological units (Turner 1950, 1969).

PROCEDURE

Male guinea pigs weighing 180–200 g are injected once daily on 4 days successively. Thyroids are re-

moved on the 6th day, weighed, and embedded for histological examination. Administration of thyrotropic hormone is followed by colloid resorption, increased vascularity, and increased epithelial cell height. Several regions of the thyroid are examined histologically. Alternatively, computer-assisted morphometry may be used. Rating scores are defined between +1 and +4.

EVALUATION

The rating scores are averaged and compared between test preparation and standard.

REFERENCES AND FURTHER READING

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N.7.5.3

Iodine Uptake

PURPOSE AND RATIONALE

This is a bioassay for thyroid activation related to the initial step of thyroid-stimulating hormone (TSH) action (Turner 1950, 1962, 1969). The uptake of iodine (trapping) as well as the release of the newly formed iodinated thyroid hormones is under the control of thyrotropin (and in animal assays, under control of hypothalamic thyrotropin-releasing hormone, TRH). As a consequence, the uptake of ^{131}I and the release of ^{131}I -labeled thyroxin are increased after administration of TSH. A method using ^{131}I release in mice has been described by McKenzie (1958) and modified by Sakiz and Guillemin (1964).

PROCEDURE

Female mice weighing 10–15 g are kept in a temperature-controlled room and fed a low-iodine diet for 10 days. They are then injected intraperitoneally with $1.5\ \mu\text{C}^{131}\text{I}$, followed 5 h later by $10\ \mu\text{g}$ L-T₄ subcutaneously. After 24 h they receive a second injection of $5\ \mu\text{g}$ L-T₄ and are used 48 h after the last injection. Under ether anesthesia 0.25 ml blood is withdrawn from the jugular vein into a heparinized syringe. Various doses of the test preparation or standard TSH are injected by the same route in 0.3 ml volume. Two hours later, again under ether anesthesia, a second 0.25-ml

sample is taken, and the radioactivity measured. The increase of radioactivity in the blood samples is dependent on the dose of TSH.

EVALUATION

A 4-point assay technique is used with six observations for each of two doses of the standard and of the unknown preparation.

MODIFICATIONS OF THE METHOD

Depletion of ^{131}I from the thyroids of chicken was used as the endpoint for a TSH assay by Bates and Cornfield (1957).

REFERENCES AND FURTHER READING

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N.7.5.4

TSH Bioassay Based on cAMP Accumulation in CHO Cells

PURPOSE AND RATIONALE

Indirect cell assays measure the activation of protein kinase A (PKA) through adenylcyclase. Several cell assays for thyrotropin using cultured FRTL-5 cells have been described (Vitti et al. 1986; Nissim et al. 1987; Horimoto et al. 1989). Persani et al. (1993) reported a cell assay for human thyrotropin using measurement of cAMP accumulation on Chinese hamster ovary (CHO) cells transfected with the recombinant TSH receptor.

PROCEDURE

Cells of the CHO-R strain JP-09 are cultured in Petri dishes in RPMI-1640 medium supplemented with 1 mM glutamine and 10% fetal calf serum. In these cells TSH biological activity is evaluated by measuring cAMP production. Cells are harvested from Petri dishes using a Trypsin-EGTA mixture and seeded in 96-well plates (10,000 cells/well). Cells are fed with fresh RPMI-1640 medium 24 h after seeding.

The assay is run after 48 h. After washing, 100 µl of TSH standard or samples diluted in hypotonic or isotonic medium containing 0.4% BSA, 10 mM HEPES, and 0.5 mM isobutylmethylxanthine, are incubated for 2 h at 37°C. Three different dilutions of immunconcentrated TSH are bioassayed in triplicate, as are TSH preparations. cAMP is measured in nonacetylated samples by an RIA method using a commercial polyclonal anti-cAMP antibody (Vitti et al. 1986).

EVALUATION

Dose–response curves or single-point comparisons are used for potency or activity estimates.

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N.7.6

Hormones Related to TSH

N.7.6.1

General Considerations

These hormonal factors are now characterized by molecular cloning. One of the characteristic symptoms of thyroid activation (Graves' or Basedow disease) is exophthalmos. A dissociation of the exophthalmos-producing activity from the TSH activity has been found (Dobyns and Steelman 1953). Moreover, time–response curves for TSH activity from the serum of patients with hyperthyroidism differed from those of the standard TSH, giving evidence for the presence of an abnormal factor, long-acting thyroid-stimulating factor (LATS). Bioassays for these factors have been developed (Ludgate 1999). TSH receptor stimulating

antibodies have also been identified and suitable animal models have been investigated (diCerbo and Corda 1999; DeFelice et al. 2004).

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N.7.6.2

Assay of Exophthalmos-Producing Substance (EPS) in Fishes

PURPOSE AND RATIONALE

Some TSH fractions were reported to produce more exophthalmos than others (Dobyns and Steelman 1953). A second fraction containing exophthalmos-producing substance (EPS) could be separated (Brunish et al. 1962). The activity of this substance was demonstrated in fishes.

PROCEDURE

Fundulus heteroclitus Linn., the common Atlantic minnow, has been found to be the suitable animal model (Albert 1945; Sobonya and Dobyns 1967; Turner 1969). Other fish species, such as *Carassius auratus* (the common gold fish; Haynie et al. 1962) and *Cyprinus carpio* (der Kinderen et al. 1960), were also used but gave less reliable results (Sobonya and Dobyns 1967). *Fundulus heteroclitus* Linn., common Atlantic minnows, 8–10 cm in length, are kept in a tank of running tap water at 10°C in winter and 18°C in summer. Before treatment the intercorneal distance is measured with Vernier calipers to the nearest of 0.1 mm. The test compound and the standard are injected intraperitoneally at various, but at least two, doses. To avoid loss of fluids by the intraperitoneal injection route, the needle is inserted into the cloaca, through the rectum, over the pelvic girdle, and into the peritoneal cavity for a distance of about 1.2 cm. The volume of fluid injected should be 0.1–0.5 ml. The effect of the hormone is to cause protrusion of the eyeballs. The intercorneal distance is measured again after 24, 48, and 72 h. The increase in proptosis is expressed

as a percentage of the intercorneal distance found at the beginning of the test.

EVALUATION

The increases of intercorneal distance after each dose are averaged and activity ratios with confidence limits calculated from the 2+2-points assay.

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N.7.6.3

Assay of Long-Acting Thyroid-Stimulating Factor (LATS) in Mice

PURPOSE AND RATIONALE

This is a modification of the TSH assay to assess the late onset of action of LATS. In the assay of McKenzie (1958) (see N.7.5.3), mice previously injected with ^{131}I show a maximum increase in serum ^{131}I after an interval of 2–3 h when TSH is administered. In the assay of serum from thyrotoxic patients, Adams (1958) noted that the maximum response in guinea pigs did not occur until after 16 h. The abnormal responses to thyrotoxicosis sera suggested the presence of an additional factor different from TSH.

PROCEDURE

Mice maintained on a low-iodine diet for 10 days are injected with $15\ \mu\text{C}$ ^{125}I and $10\ \mu\text{g}$ Na L- T_4 . Four days later, 0.1 ml of blood is obtained by retro-orbital puncture immediately before the injection of the test substance 2 and 9 h later. Radioactivity in the blood is then measured. By definition, radioactivity which is maximal after 2 h is indicative of TSH, whereas LATS causes a maximal increase at 9 h.

EVALUATION

The increases of radioactivity after 2 and 9 h are compared and evaluated by statistical methods.

MODIFICATIONS OF THE ASSAY

Ikeda and Nagataki (1983) and Ikeda et al. (1984) used male DDY mice weighing 15 g. They were fed a low-iodine diet for 14 days and then injected daily with $1\ \mu\text{g}$ of 3,5,3'-triiodothyronine (T_3) s.c. and given T_3 ($5\ \mu\text{g}/\text{ml}$) ad libitum in drinking water until sacrifice. From the 5th day of T_3 treatment they were injected i.p. with 0.25 ml of LATS-positive serum for 9 days. Groups of five mice were sacrificed before, 1, 3, 5, 7, and 9 days after the first injection of LATS. Then $1\ \mu\text{Ci}$ ($0.5\ \text{ml}$) of Na ^{131}I was administered i.p. 1 h before sacrifice. Thyroid lobes were excised, weighed, and radioactivity was measured by a gamma counter. Immediately before injection of Na ^{131}I , approximately $60\ \mu\text{l}$ of blood was collected from the orbital plexus with heparinized capillary tubes and centrifuged at 12,000 rpm for 3 min. T_4 concentrations in serum were determined by radioimmunoassay.

Ealey et al. (1984, 1985) developed a sensitive cytochemical bioassay for thyroid stimulators, using reference preparations of thyrotropin and LATS. Thyroid stimulators cause changes in lysosomal membrane permeability within the thyroid follicular cells of guinea pigs, which can be monitored by measuring increased intralysosomal enzyme activity (in the case of this assay, naphthylamidase), with a chromogenic substrate leucine-2-naphthylamide, which itself does not readily permeate the lysosomal membrane in unstimulated cells.

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N.8

Posterior Pituitary Hormones

N.8.0.1

General Considerations

Oxytocin and vasopressin were the first hormones isolated from the posterior pituitary lobe (Dale and Laidlaw 1912; Hogben et al. 1924; Fromherz 1926; Schauermann 1937). Vasopressin has antidiuretic activity and, at higher doses, hypertensive effects as well as endocrine functions (Hedge and Huffman 1987) and effects on the central nervous system (Gash et al. 1987), which are however outside the scope of this chapter. In terms of bioassays, the antidiuretic activity is the main parameter. Many analogs of vasopressin have been synthesized resulting in selective agonists and antagonists (Vogel and Hergott 1963; Allison et al. 1987; Mah and Hofbauer 1987; Manning et al. 1987). Different types of vasopressin receptors have been identified: V_1 (V_{1a} , V_{1b}) and V_2 -receptors (Jard et al. 1976, 1986; Fahrenholz et al. 1988; Walker et al. 1988; Burnatowska-Hledin and Spielman 1989). Research on the vasopressin analogs has provided several compounds of clinical utility, and antagonists of vasopressin are now increasingly explored for several indications, including nonpeptide vasopressin antagonists (Mayinger and Hensen 1999; Serradeil-Le Gal et al. 2002; Greenberg and Verbalis 2007; Urban et al. 2007).

The use of bioassays in the evaluation of these vasopressin and oxytocin analogs has been reviewed (Liard 1988; Chan et al. 2000).

Oxytocin receptors have been described in several organs, such as uterus, mammary gland and CNS (Soloff 1976; Hruby and Chow 1990).

The synthesis and development of several orally active, nonpeptide oxytocin antagonists have been reported (Bell et al. 1998; Kuo et al. 1998).

Radioimmunoassays are available for both oxytocin (Kagan and Glick 1978) and vasopressin (Glick and Kagan 1978).

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N.8.1

Oxytocin

N.8.1.1

Isolated Uterus

PURPOSE AND RATIONALE

Several authors, such as Dale and Laidlaw (1912), Fromherz (1926), Glaubach and Molitor (1932), Lipschitz and Klar (1933), and Simon (1933), used the isolated uterus of virgin guinea pigs as a sensitive test for determination of oxytocin activity. The isolated uterus of the rat (Holton 1948) is less sensitive but, in contrast to the guinea pig, the rat uterus shows no spontaneous contractions in solutions with low calcium and glucose concentrations. Historically, the method has been adopted by several pharmacopoeias, e. g., by the British Pharmacopoeia (1988). The United States Pharmacopoeia 23 (1995) uses the isolated guinea pig uterus for determination of oxytocin activity in vasopressin preparations. Physicochemical assays are now used for standardizing drug content, instead of the biological responses.

PROCEDURE

Female Sprague-Dawley or Wistar rats weighing 120–200 g are used. At 18–20 h prior to the assay, the rat is injected i.m. with 100 µg of estradiol benzoate for priming (receptor induction). Immediately before the assay the rat is tested for estrogen-

induced epithelial proliferation by vaginal smear. One horn of the uterus is suspended in an organ bath containing a solution of the following composition:

| | |
|----------------------------------|----------|
| Sodium chloride | 6.62 g/l |
| Potassium chloride | 0.45 g/l |
| Calcium chloride | 0.07 g/l |
| Sodium hydrogen carbonate | 2.56 g/l |
| Disodium hydrogen orthophosphate | 0.29 g/l |
| Sodium dihydrogen orthophosphate | 0.03 g/l |
| Magnesium chloride | 0.10 g/l |
| D-Glucose | 0.50 g/l |

The bath temperature is maintained at 32°C, a temperature at which spontaneous contractions of the uterus are abolished and the preparation maintains its sensitivity. The solution is bubbled with a mixture of 95% O₂ and 5% CO₂. The preparation is loaded with 1–2 g and the contractions recorded using a Statham transducer and a polygraph. Two doses of the standard preparation are added, ranging usually between 10 and 50 µU/ml organ bath. The preparation examined is diluted in such a way to obtain responses on the addition of two doses similar to those obtained with the standard preparation. The ratio between the two doses of the preparation being examined should be the same as that between the two doses of the standard preparation. The two doses of the standard preparation and the two doses of the preparation being examined are given according to a randomized block or a Latin square design and at least six to eight responses to each are recorded. The doses should be recorded at regular intervals of 3–5 min depending on the rate of recovery of the muscle.

EVALUATION

The activity and potency ratios with confidence limits are calculated from the 2+2-points assay.

MODIFICATIONS OF THE METHOD

The isolated rat uterus is also used to test spasmolytic activity of various drugs against oxytocin as spasmogen. Liebmann et al. (1993) used the rat uterus to test the pharmacological and molecular actions of the bradykinin B₂ receptor antagonist, Hoe 140.

The method described by Schübel and Gehlen (1933) using the **uterus of cats** 2–4 days after partum is of historical interest only.

In addition to the isolated rat uterus, Berde et al. (1957) used the **rat uterus in situ**, the **cat uterus in**

vitro, and the **cat uterus in situ** for evaluation of synthetic analogs of oxytocin.

Murray and Miller (1960) observed characteristic post-urial changes in rats following administration of oxytocin to unanesthetized rats described as “cramping” which was dose-dependent in estrogen-pretreated animals.

An *in vitro* **hen oxytocic assay** was designed by Munsick et al. (1960). Muscle strips of the uterine portion of the oviduct of laying hens are dissected and suspended in a van Dyke-Hastings solution containing 0.15 mM calcium, 0.5 mM magnesium and 100 mg% glucose. The strips are 2–3 cm long and 2–3 mm wide. The solution is gassed with oxygen containing 5% carbon dioxide and maintained at a temperature of 43°C. These conditions are necessary to prevent spontaneous contractions.

The **isolated uterus from immature guinea pigs** was used for evaluation of oxytocin activity by Fromherz (1926) and by Vogel and Hergott (1963).

Guissani et al. (1995) and Pettibone et al. (1996) reported the effect of oxytocin antagonists in **pregnant rhesus monkeys in vivo**.

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N.8.1.2

Chicken Blood Pressure

PURPOSE AND RATIONALE

Oxytocin induces a transient fall in blood pressure in chicken and other birds. This effect can be used as an assay for oxytocin (Coon 1939). The method has been modified by Munsick et al. (1960) and accepted by pharmacopoeias; for example, the United States Pharmacopoeia 23 (1995a, 1995b) uses the chicken blood pressure method for determination of oxytocin activity

with three doses of standard and test preparation and calculation of the activity ratio with confidence limits.

PROCEDURE

White Leghorn chickens weighing 1.2–2.0 kg are anesthetized by intravenous injection with 200 mg/kg sodium phenobarbital via the brachial vein. The ischiadic artery is exposed by removing the feathers from the outer surface of the left thigh, and an incision 7–8 cm long is made in the skin, parallel to and about 1.5 cm below the femur, exposing the gluteus primus muscle. The lower edge of this incision is retracted to expose the edge of the gluteus primus muscle overlying the semitendinosus muscle. The edge is then freed for the length of the incision, and when the free edge is lifted, the ischiadic artery, the ischiadic vein, and the crural vein can be seen lying along the edge of the semitendinosus muscle. The gluteus primus muscle is cut at right angles near the proximal end of the incision and the resulting flap deflected and secured to the upper thigh. A length of the ischiadic artery and the crural vein are dissected free and the artery is cannulated. The cannula is connected to a Statham pressure transducer. Blood pressure should be between 100 and 120 mmHg. The crural vein is cannulated for injections of the test preparations. Intravenous injection of oxytocin induces in chickens an immediate, transient fall in blood pressure. Doses of the standard are chosen which are followed by a decrease of blood pressure of between 20 and 40 mmHg. The required doses normally lie between 20 and 100 mU.

Two doses of the standard and two doses of the test preparations are injected according to a randomized block or to a Latin square design and at least six to eight responses to each should be recorded. The interval between injections should be constant and lies between 3 and 10 min, depending on the rate at which the blood pressure returns to normal.

EVALUATION

The responses to each dose are averaged and potency ratios with confidence limits calculated from the 2+2-points assay.

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N.8.1.3

Milk Ejection in the Lactating Rabbit or Rat

PURPOSE AND RATIONALE

A sensitive bioassay for the estimation of oxytocin was described by van Dyke et al. (1955). This test makes use of the milk-ejecting properties of oxytocin.

PROCEDURE

A rabbit in the first or second week of lactation is anesthetized with urethane and pentobarbital. Usually, artificial respiration is not necessary. One jugular vein is cannulated for injections. One of the six ducts in a nipple of the rabbit is cannulated with a hypodermic needle and connected with a Statham strain gauge transducer. Two doses of the standard and the test preparation are injected according to a randomized block or to a Latin square design and at least six to eight responses to each should be recorded. The interval between injections should be constant at 3 min.

EVALUATION

The responses of each dose are averaged and potency ratios with confidence limits calculated from the 2+2-points assay.

MODIFICATIONS OF THE METHOD

The British Pharmacopoeia (1988) recommends the measurement of milk-ejection pressure in the lactating rat. A lactating rat weighing about 300 g in the 3rd to 21st day after parturition is anesthetized by pentobarbitone sodium. The trachea is cannulated. One jugular or femoral vein is cannulated for injection of the test preparations. The tip of one lower inguinal teat is excised and a polyethylene tube with an external diameter of 0.6 mm is inserted to a depth sufficient to ob-

tain appropriate measurement of pressure into the primary teat duct which opens onto the cut surface and is tied firmly in place with a ligature. The cannula is connected with a suitable strain gauge pressure transducer for recording on a polygraph.

Tindal and Yokoyama (1962) recommended the use of guinea pigs using essentially a similar procedure, but the injection is made into the internal saphenous artery after ligation of the main branches supplying the limb. In the guinea pig the mammary glands are supplied with blood from the external pudendal arteries, which branch from the internal saphenous artery just as they enter the legs.

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N.8.1.4

Oxytocin Receptor Determination

The family of the vasopressin and oxytocin receptors has been studied very intensely (Peter et al. 1995) in order to obtain evidence for new indications, and to determine the specific regions in each receptor subtype and the associated effector systems for the G-protein-linked subtypes (Thibonnier et al. 1998).

PURPOSE AND RATIONALE

Premature labor and pre-term delivery is an important cause of death among infants and a major cause of newborn and child morbidity. Strong evidence exists that oxytocin and catecholamines are involved in the spontaneous uterine contractions which bring about premature delivery. Development of oxytocin antagonists is believed to be of therapeutic value for the pre-

vention of pre-term labor (Manning et al. 1995; Chan et al. 1996).

In vitro binding to the oxytocin receptor is used as the first step in the characterization of potential oxytocin antagonists (Pettibone et al. 1990, 1991, 1993a, 1993b, 1996; Evans et al. 1993; Manning et al. 1995; Freidinger and Pettibone 1997; Pettibone and Freidinger 1997).

PROCEDURE

Uterine tissue is taken from nonpregnant adult Sprague-Dawley rats pretreated (18–24 h) with diethylstilbestrol (300 µg/kg, i.p.) and mammary tissue from lactating rats (4–14 days of lactation). The tissues are homogenized in 10 mM Tris containing 1 mM EDTA and 0.5 mM dithiothreitol, pH 7.4 and centrifuged at 48,000 g for 30 min at 4°C. The resulting pellets from mammary/uterine tissue are resuspended in 50 mM Tris/5 mM MgCl₂/0.1% BSA (pH 7.4) and centrifuged again to produce the final pellet. Competition studies are conducted at equilibrium for 60 min at 22°C using 1 nM [³H]OT (30–60 Ci/mmol, New England Nuclear, Boston, Mass., USA) in the following buffer: 50 mM Tris/5 mM MgCl₂/0.1% BSA (pH 7.4). Nonspecific binding (5%–10% of total binding) is determined using 1 µM unlabeled oxytocin. *IC*₅₀ values are calculated from the linear regression analysis of log concentration of inhibitor versus percentage inhibition of specific binding. Saturation binding studies are conducted at equilibrium using a 100-fold range of radioligand concentrations (i.e., 0.1–10 nM) and analyzed by a nonlinear regression program (McPherson 1985a, 1985b). The binding reactions are initiated by the addition of the tissue preparation (final protein concentrations, 100–200 µg protein/ml) and terminated by rapid filtration through Skatron glass fiber filters using a Skatron cell harvester system (Model 7019, Skatron, Sterling, Va., USA).

EVALUATION

Inhibition constants (*K*_i) are calculated for each compound from three to six separate *IC*₅₀ determinations ($K_i = IC_{50} / [1 + c / K_d]$) using mean dissociation constants (*K*_d) obtained from saturation binding assays.

MODIFICATIONS OF THE METHOD

Maggi et al. (1994) used cultured Hs 805.Ut (corpus uteri, normal, human) cells or cells obtained from women in the early follicular or late luteal phase to study binding of antagonists at the human oxytocin receptor.

Pak et al. (1994) compared the binding affinity of oxytocin antagonists to human and rat oxytocin receptors and correlated the results with the rat oxytocic bioassay.

Species differences in central oxytocin receptor gene expression were investigated by Young et al. (1996).

Cloning and expression of the rhesus monkey oxytocin receptor was reported by Salvatore et al. (1998).

Molecular cloning and functional characterization of the oxytocin receptor from a rat pancreatic cell line (RINm5F) was reported by Jeng et al. (1996).

Elands et al. (1987) and Klein et al. (1995) recommended selective radioligands for the oxytocin receptor for the structure–activity research directed to receptor subtypes.

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N.8.2

Vasopressin

N.8.2.1

Hereditary Vasopressin Deficiency in Rats (Brattleboro Strain)

This bioassay is based on an animal strain with genetic deficiency of vasopressin synthesis. Patients with diabetes insipidus excrete large amounts of very diluted urine and need a high fluid intake. This disorder is

due to a lack of vasopressin. A similar syndrome has been found in Brattleboro rats. In the rare nephrogenic form, the vasopressin receptors are defective (Sokol and Valtin 1982; Nyunt-Wai and Laycock 1990; Szot and Dorsa 1992). Vasopressin is also considered to be a modulator in the central nervous system in particular learning and memory processes. Therefore, many studies on learning and memory have been performed with this strain of rats. Valtin et al. (1965) found very little vasopressin in hypothalami and pituitaries of homozygous Brattleboro rats.

Schmale and Richter (1984) and Schmale et al. (1984) found a single base deletion in the vasopressin gene as the cause of diabetes insipidus in Brattleboro rats. The mutant vasopressin gene is transcribed but the message is not efficiently translated. Spontaneous hypertensive rats crossbred with Brattleboro rats inherit the mutated vasopressin gene (McCabe et al. 1988). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus can be reversed by a vasopressin agonist (Laycock et al. 1994)

For testing aquaretic effects in Brattleboro rats see C.1.2.1.

MODIFICATIONS OF THE METHOD (OTHER DIABETES INSIPIDUS MODELS)

Similar studies were reported by: Byrnes et al. 1953; Randall and Selitto 1958; Eviatar et al. 1961; Turner 1965; Dorfmann 1969; Neri et al. 1972; Neumann et al. 1977; Furr et al. 1987; Snyder et al. 1989; Christiansen et al. 1990; Shibata et al. 1992; Neubauer et al. 1993; Tagekawa et al. 1993

Several other animal models of diabetes insipidus have been developed and characterized (Herman et al. 1986; Grant 2000; Lloyd et al. 2005; Petersen 2006) for the understanding of pathophysiology, for explanation of genetic deficiencies, and to validate the animal models based on substitution of vasopressin deficiency.

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N.8.2.2

Vasopressor Activity

PURPOSE AND RATIONALE

Vasopressin activity can be determined by the increase of blood pressure observed after surgical or pharmacological elimination of the central and peripheral nervous regulation of the cardiovascular system. Dekansky (1952) described a quantitative assay of vasopressin in anesthetized rats after blockade of other pressor substances by dibenamine (pithed rat).

PROCEDURE

A male Wistar rat weighing about 300 g is anesthetized by subcutaneous injection of 1.75 g/kg urethane. After 45–60 min, the trachea is cannulated with a polyethylene tube of 2.5 mm diameter. One femoral vein is cannulated for injections and the carotid artery for measuring blood pressure with a Statham transducer. The central and peripheral nervous systems, including both vagi and associated sympathetics, are left intact. No artificial respiration is necessary. Heparin (2000 U/kg) is injected through the venous cannula and washed through with saline. [Dibenamine *N*-(2-chloroethyl)dibenzylamine hydrochloride] is injected twice intravenously with an interval of 10 min at a dose of 1 mg/kg. The blood pressure stabilizes at a basal level of about 50 mmHg. Small doses of vasopressin induce an increase in blood pressure which is depen-

dent on the dose. Two doses of vasopressin standard (approximately 3–5 mU) and two doses of test preparation are injected repeatedly (usually 6 times) using a Latin square design. The doses are injected at intervals of 10–15 min.

EVALUATION

The responses of each dose are averaged and potency ratios calculated from the 2+2-points assay.

MODIFICATIONS OF THE METHOD

One of the first recommendations for standardizing posterior pituitary extract was by measuring blood pressure in anesthetized dogs (Hamilton 1912).

Vogel and Hergott (1963), studying the properties of a synthetic vasopressin analog, described a method in decerebrate rabbits previously used in this laboratory for standardization of posterior pituitary extracts. Rabbits weighing 2–3 kg were anesthetized by slow intravenous injection of butallylonal. The trachea is cannulated and the cannula connected with a respiration pump. One femoral vein is cannulated for injection of the test compounds. One carotid artery is cannulated for measurement of blood pressure with a Statham transducer. For chemical decapitation, the head of the anesthetized rabbit is bent forwards and a needle introduced into the foramen occipitale magnum, and then 0.5 ml of 30% trichloroacetic acid is injected. Artificial respiration is started immediately. Blood pressure is stabilized at a level of 30–40 mmHg. Two doses of standard and of test preparation are injected according to a Latin square design. The method has been proven to be very sensitive.

The British Pharmacopoeia (1988) recommends the intravenous injection of 10 mg/kg of the α -adrenoreceptor blocking agent phenoxybenzamine hydrochloride 18 h prior to the experiment to stabilize the blood pressure at a low level. USP 23 (1995a, 1995b) uses two doses of standard and test preparation in the phenoxybenzamine-blocked rat for calculation of vasopressin activity with confidence intervals in vasopressin and oxytocin preparations.

Knape and van Zwieten (1988) used the pithed rat to study vasoconstrictor activity of vasopressin after pretreatment with various drugs.

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N.8.2.3

Antidiuretic Activity in the Conscious Rat

PURPOSE AND RATIONALE

For some period of time, it was uncertain whether vasopressin and adiuretin are the same or separate hormones. Special tests for antidiuretic activity in water-loaded rats were developed (Burn 1931) and adopted by pharmacopoeias. Vasopressin analogs show considerable dissociation of vasopressive and antidiuretic activities (Vogel and Hergott 1963).

PROCEDURE

Wistar or Sprague-Dawley rats weighing between 140 and 250 g of either sex are used. The range of weights in any one test should not exceed 50 g. Not less than 2 days before testing the preparation, the rats have to be accustomed to the metabolism cage by carrying out a preliminary test. All animals are injected with 0.1 ml saline solution per 100 g body weight instead of the test preparations given in the main test. Any rat that shows signs of stress or undue excitement or that has abnormally low or high rate of urine excretion should not be used in the main test. Food and water are withheld during each test but access is allowed between the tests. The rats are assigned at random to four groups,

each no less than four animals, and are weighed and marked for identification purposes. By stomach tube each rat receives a volume of water warmed to approximately 37°C and equivalent to 5% of the animal's body weight. Each rat is placed in a separate cage for collection of urine. Then 30 min later, the volume excreted by each rat is recorded and a second volume of tap water equal to the volume of urine together with a further volume equivalent to 3% of the animal's body weight is administered. This provides a total water load equivalent to 8% of the animal's body weight.

Using a different group of rats and two dilutions of the preparation to be examined and of the standard preparation, immediately after administration of the second dose of water, each rat is injected subcutaneously with a volume of the appropriate dilution equivalent to 0.1 ml per 100 g body weight. The urine passed during the first 5 min after injection is discarded and the volumes collected at intervals of 15 min are noted until a volume greater than 30% of the total water load is excreted.

EVALUATION

The responses of each dose are averaged and activity ratios with confidence limits are calculated from the 2+2-points assay.

MODIFICATIONS OF THE METHOD

Hydrated **conscious dogs** have been used to test the antidiuretic activity of vasopressin by van Dyke et al. (1955).

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N.8.2.4

Antidiuretic Activity in the Rat in Ethanol Anesthesia

PURPOSE AND RATIONALE

This is a sensitive bioassay often reported for vasopressin analogs, which has been applied extensively but is no longer required due to the availability of other methods (e. g., Brattleboro rats). Ethanol suppresses the secretion of endogenous antidiuretic hormone (van Dyke and Ames 1951). Sensitive methods measuring urine output in ethanol-induced anesthesia were described (Dicker 1953; Dettelbach 1958; Munsick et al. 1960; Berde and Cerletti 1961), whereby the water load is automatically kept constant.

PROCEDURE

Female Sprague-Dawley rats weighing 200–250 g are spayed 7–10 days prior to use in order to eliminate the effect of gonadal steroids on the response to a water load. Each animal is given a 5-ml gavage of tepid tap water. At 3-day intervals thereafter, the water load is increased by 1-ml increments until a 10-ml level is reached. The day before an assay is to be performed, two or three animals are selected and given a 10-ml gavage of 2% ethanol. The animals are fasted overnight but are allowed free access to water. On the test day, they are placed into individual metabolism cages for water loading and anesthetizing. Each rat receives 5% of its body weight of warm 12%–15% ethanol by stomach tube. This induces anesthesia within a few minutes. After 45 min the same volume of 2% ethanol is again given by gavage. After 1 h the bladder is emptied by suprapubic pressure and the total urine output is measured.

The animal with the largest volume is selected and the water load calculated as the total volume administered less the volume excreted. The load on this animal is now increased to between 6% and 8% of its body weight with warm 2% ethanol. Then the urinary bladder is catheterized. Polyethylene tubing is inserted into the rat's stomach. A femoral vein is cannulated for injections. The hind legs are secured to the operation table, while the front legs are left free. Assays are started when urine flow reaches a steady level of at least 50 µl/min. The catheter from the bladder is connected to an apparatus consisting of two Woulff bottles. Urine is directed through the catheter into the first Woulff bottle, displacing fluid into the second Woulff bottle. The second bottle is filled with 0.5% NaCl solution in 50% ethanol to reduce the size of the drops. Each drop activates an impulse counter. The drops are collected in a small glass reservoir and led by means

of polyethylene tubing to the urine metering pipette. When the fluid level in this tube rises and makes contact with an adjustable needle electrode near the top, another pipette which is calibrated to the same volume empties a solution of 5% glucose in 2% ethanol through the gavage tube into the rat's stomach. By these means a constant water load is maintained.

The animals produce urine at a relatively steady rate for 3–5 h. Intravenous injections of vasopressin produce characteristic changes (decreased rate of urine flow), which begin usually within 2 min and subside in most instances within 15 min. The range in which the assay of antidiuretic activities is useful is between 2 and 64 μ U/injection.

EVALUATION

Antidiuretic potency is calculated as the log ratio of the volume excreted in 5 min preceding injection to the volume excreted in the 5 min beginning 1 min after injection. Dose–response relationships are established for the standard and the test preparation and potency ratios are calculated.

MODIFICATIONS OF THE METHOD

Berde and Cerletti (1961) placed the rat anesthetized with ethanol on a balance. The water loss due to urine excretion and perspiration insensibilis is replaced automatically using a solution of 5% glucose in 2% ethanol.

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N.8.2.5

Spasmogenic Activity of Vasopressin in the Isolated Guinea Pig Ileum

PURPOSE AND RATIONALE

The ileum of the guinea pig has been found to be quite sensitive to vasopressin (Simon 1933; Schaumann 1937; Vogel and Hergott 1963). This is a direct

effect on smooth muscle similar to the pressor effect. This test is not specific for vasopressin.

PROCEDURE

Pieces of guinea pig ileum are suspended in an organ bath according to the Magnus' technique (see J.4.3.1). Two different doses of the test preparation and of the standard are applied according to a Latin square design and the contractions measured using a strain gauge transducer. Four to six doses of each solution are measured.

EVALUATION

The responses of each dose are averaged and activity ratios with confidence limits are calculated from the 2+2-points assay.

REFERENCES AND FURTHER READING

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N.8.2.6

Vasopressin Receptor Determination

PURPOSE AND RATIONALE

As in other areas of drug research, when specific receptor preparations became available for *in vitro* research, those identifying new vasopressin-like compounds (in structure–activity studies) turned to new methods with high specificity and capacity, replacing the cumbersome bioassays. Tests for receptor affinity are also used in “receptor screens” for the general pharmacology of new drug candidates, to predict their spectrum of activities.

Arginine vasopressin exerts its action through three membrane-bound G-protein-coupled receptor subtypes (V_{1a} , renal V_2 and V_3). The vasopressin-induced antidiuresis (via V_2 receptors coupled to aquaporins) helps maintain plasma osmolality and salt (NaCl) balances. The human V_1 , V_2 and V_3 (V_{1b}) receptors and water-selective membrane proteins (aquaporins) in the kidney have been cloned, and receptor signaling has been characterized in detail (Thibonnier et al. 1998).

Pharmacological characterization of the human vasopressin receptor subtypes stably expressed in Chi-

nese hamster ovary cells was reported by Tahara et al. (1998).

Selective nonpeptide vasopressin antagonists (without agonistic activity) have been shown to be aquaretic agents in animals and humans. The development and therapeutic indications of orally active vasopressin receptor antagonists were reviewed by Thibonnier (1998), as were the postreceptor signaling mechanisms for the vasopressin receptor subtypes.

Serradeil-Le Gal et al. (1993, 1994a) studied the biochemical and pharmacological properties of a nonpeptide antagonist on rat and human vasopressin V_{1a} receptors, to explore the possibility of using centrally acting derivatives for indications in neuropharmacology.

PROCEDURE

Tissue samples from human uterus, adrenals, kidneys, and pituitaries are collected in conformity with national ethical rules. Uterus, adrenal, and kidney samples are immediately chilled in cold saline. Membranes are prepared within 3 h after collection. Pituitaries are collected within 6 h after death and immediately frozen in liquid nitrogen. Bovine kidneys can be obtained from a local slaughterhouse. Rat mammary tissue is taken from 19-day Sprague-Dawley pregnant rats.

For human adrenal membrane preparations, pieces of adrenal glomerulosa zone are suspended in a cold buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, PMSF) and fragmented using a glass/glass Dounce homogenizer. The homogenate is filtered through glass wool and centrifuged for 15 min at 1500 g at 4°C. The pellet is incubated for 15 min in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF) and kept on ice to allow cell lysis. Lysed cells are recovered by centrifugation at 1500 g for 15 min at 4°C, homogenized using a loose-fitting Dounce homogenizer in hypotonic buffer supplemented with 40% glycerol, and stored at -20°C. Before experiments, glycerol is eliminated by washing the membranes in glycerol-free hypotonic buffer.

For preparation of hypophyseal membranes, frozen entire pituitary glands are rapidly thawed at 37°C in isotonic buffer supplemented with 0.1 mM PMSF, and the adenohipophyses are separated. Adenohipophyseal membranes are prepared as described above for adrenal membranes.

For rat mammary gland membrane preparations, tissues are minced and homogenized in 50 mM 10%

(wt/vol) buffer A Tris-HCl, pH 7.4, 320 mM sucrose, and 0.5 mM dithiothreitol and centrifuged at 900 g for 15 min; the pellet is resuspended in buffer A and centrifuged as above. The two 900-g supernatants are filtered through cheesecloth and centrifuged at 70,000 g for 20 min. The pellet is washed with buffer B containing 50 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$. Finally, the 70,000-g washed pellet is suspended in buffer B at a final concentration of ~8 mg protein/ml and stored in aliquots in liquid nitrogen.

V_{1a} binding assays using ^{125}I linear AVP antagonist on human adrenal membranes, myometrial membranes from nonpregnant uterus, or platelet membranes are performed using an incubation medium (200 μ l) containing 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA), 0.05 mg/ml soy bean trypsin inhibitor, 0.5 mg/ml bacitracin, 0.1 mM PMSF, 0.5 mM EDTA, 10–60 pM ^{125}I -AVP linear antagonist, and increasing amounts of test compound. The reaction is started by the addition of membranes (10–40 μ g/assay) that are incubated at 30°C for 45 min. The reaction is stopped by adding 3 ml of ice-cold filtration buffer (10 mM Tris HCl, pH 7.4, and 3 mM $MgCl_2$) followed by filtration through GF/C Whatman glass microfiber filters that have been soaked for at least 5 h in a solution containing 10 mg/ml BSA. Filters are washed five times with 3 ml of filtration buffer and counted for radioactivity by gamma spectroscopy. Nonspecific binding is determined in the presence of 0.3 μ M unlabeled iodinated AVP linear antagonist or 1 μ M AVP.

EVALUATION

The IC_{50} value is defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding. Inhibition constant (K_i) values are calculated from the IC_{50} values using the Cheng and Prussoff equation. Data for equilibrium binding (K_d , B_{max}), competition experiments (IC_{50} n_{Hill}), and kinetic constants (k_{obs} , k_1) are analyzed using an iterative nonlinear regression program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

A receptor assay for arginine-vasopressin is described by Gopalakrishnan et al. (1986).

Pearlmutter et al. (1985) characterized a specific binding site for arginine-vasopressin in particulate membranes from rat aorta.

Pávó et al. (1993) reported the synthesis and binding characteristics of two sulfhydryl-reactive probes for vasopressin receptors.

Serradeil-Le Gal et al. (1994b) tested the effect of a nonpeptide vasopressin V_{1a} vasopressin antagonist on the binding and mitogenic activity of vasopressin in Swiss 3T3 cells.

Yatsu et al. (1997) tested the vasopressin antagonistic activities of a nonpeptide dual vasopressin V_{1a} and V_2 receptor antagonist on V_{1a} receptors in dog platelets and on V_2 receptors in dog kidney homogenates.

Ogawa et al. (1996) and Tahara et al. (1997a) tested V_{1a} receptor binding in homogenates of rat liver and V_2 receptor binding in rat kidney.

Barberis et al. (1995) characterized a linear radioiodinated vasopressin antagonist as an excellent radioligand for vasopressin V_{1a} receptors.

Radioligands for vasopressin V_1 receptors were described by Elands et al. (1988) and by Kelly et al. (1989).

Ala et al. (1997) reported the properties of a radioiodinated antagonist for human vasopressin V_2 and V_{1a} receptors and recommended this ligand for further studies on human vasopressin V_2 receptor localization and characterization, when used with a selective vasopressin V_{1a} ligand.

Howl and Wheatley (1995) found species heterogeneity in the characteristics of V_{1a} receptors and in the expression of hepatic V_{1a} receptors.

Thibonnier et al. (1994) reported molecular cloning, sequencing, and functional expression of a cDNA encoding the human V_{1a} vasopressin receptor.

Carnazzi et al. (1997) described photoaffinity labeling of the rat V_{1a} vasopressin receptor using a linear azidopeptidic antagonist.

Phalipou et al. (1997) studied the peptide-binding domains of the human V_{1a} vasopressin receptor with a photoactivatable linear peptide antagonist.

DDAVP (1-desamino-8-D-arginine vasopressin), which is considered to be a standard V_2 vasopressin receptor-selective agonist, was found to act also as an agonist at the V_{1b} vasopressin receptor (Saito et al. 1997).

Tahara et al. (1997b) investigated the effects of a nonpeptide V_{1a} and V_2 vasopressin receptor antagonist in binding and functional studies of rat vascular smooth muscle cells.

Tahara et al. (1998) expressed three subtypes of human arginine-vasopressin receptors, hV_{1a} , hV_{1b} , and hV_2 , in Chinese hamster ovary cells and characterized them by [3 H]AVP binding. In addition, the coupling of the expressed receptor protein to a variety of signal transduction patterns was investigated.

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- Cloning, characterization, and transcriptional regulation of the TRH gene are reviewed by Wilber and Ai-Hua-Xu (1998).
- Pekary (1998) discussed the physiological role of TRH-enhancing peptide (Ps4) which results from proteolytic processing of prepro-TRH.
- Effects of TRH and its analogs in the CNS not related to the release of thyroid-stimulating hormone (TSH) (extrapituitary effects) have been found, indicating perhaps other therapeutic indications (Metcalf 1983; Flohé et al. 1983; Nemeroff et al. 1984; Horita et al. 1986; Horita 1998).
- Radioimmunoassays for TRH are available (Bassiri et al. 1978).
- Furukawa et al. (1980) reported the local effects of TRH on the isolated small intestine and tenia coli of the guinea pig. This effect could be related to the local release of catecholamines.

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N.9

Hypothalamic Hormones

N.9.1

Thyrotropin-Releasing Hormone (TRH)

N.9.1.1

General Considerations

Hypothalamic regulatory hormones are used in diagnostic procedures and for therapy, usually modified by chemical synthesis to enhance activities (Reichlin

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N.9.1.2

TRH Receptor Binding Assays

PURPOSE AND RATIONALE

Thyrotropin-releasing hormone (TRH) receptors from mouse (Straub et al. 1990; Jones et al. 1996), from rat (Sellar et al. 1993), and from men (Duthie et al. 1993; Matre et al. 1993; Yamada et al. 1993; Hinuma et al. 1994) have been expressed and characterized. Two isoforms have been identified (de la Peña et al. 1992; Lee et al. 1995). Constitutive activity of native TRH receptors has been demonstrated by Jinsi-Parimoo and Gershengorn (1997). Molecular and cellular biology of TRH receptors has been reviewed by Vassart and Dumont (1992) and by Gershengorn and Osman (1996).

TRH receptors have been localized in the pituitary gland (hypothalamic control of thyroid-stimulating hormone secretion), and in other brain regions (extrapituitary effects), which have been extensively explored but not resulted in therapeutic applications (Burt and Taylor 1983). TRH receptors can be determined by binding of [³H]MeTRH (Taylor and Burt 1981; Sharif and Burt 1983; Jarowska-Feil et al. 1995; Yamada et al. 1995) or [³H](3-Me-His²)TRH (Simasko and Horita 1982).

TRH receptor affinity methods can be applied to the investigation of molecules which might interact with these receptors (Faden et al. 2005; Colson and Gershengorn 2006), and to the quantitate the description of receptor changes *ex vivo* after therapy.

PROCEDURE

Pooled tissue samples are homogenized in a sodium phosphate buffer (20 mM, pH 7.4) and centrifuged at

30,000 g for 30 min. The resultant pellets are washed twice by means of resuspension and centrifugation. The washed membranes are dispersed in fresh buffer and are used for the TRH receptor binding assay.

The membranes are incubated in 250 μ l of the total volume with 0.5–8 nM of [³H]MeTRH (NEN, sp. act. 62.8 Ci/mmol), in the presence or absence of 10 μ M of TRH for 5–6 h at 0°C (in a water-ice bath). The receptor-bound and free [³H]MeTRH are separated by rapid filtration through a glass fiber filter GF/B (Whatman) under reduced pressure (Harvester-Brandel). The trapped receptor-bound radioactivity is determined by liquid scintillation spectrometry (Beckman). The amount of specifically bound [³H]MeTRH is expressed as fmoles per mg of protein.

EVALUATION

The data are subjected to the 6-point Scatchard analysis. The receptor density (B_{max}) and apparent dissociation constant (K_d) are determined.

MODIFICATIONS OF THE METHOD

Sun et al. (1998) described cloning and characterization of the chicken TRH receptor.

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- ding and Schally 1969). This is a historical test based on the effect of thyroid hormone release by TRH (indirect bioassay). The rise in radioactivity in blood 2 h after injection of the TRH preparations is proportional to the effect of TSH released by TRH. It needs to be assessed whether the extract being tested is free of TSH contamination.

PROCEDURE

Weanling mice of the Swiss-Webster strain weighing 15 g are fed a low-iodine diet and given distilled water for 10 days. At time zero, 4 μC Na¹³¹I, 1 μg thyroxine, and 1 mg codeine phosphate are injected subcutaneously. Codeine is given again subcutaneously at 24, 30, and 48 h after time zero and then 2 h after the fourth injection of codeine, blood is taken from the orbital venous sinus. The test preparation or TRH standard is injected intravenously at increasing doses (0.01, 0.03, 0.09 $\mu\text{g}/\text{mouse}$), and 2 h later, a second blood sample is taken from the orbital venous sinus. The response is obtained by the increase of radioactivity from the first to the second blood sample (Δcpm).

EVALUATION

Dose–response curves are established for the test preparation and standard in order to calculate potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

TRH also stimulates release of TSH and subsequently of ^{131}I in rats pretreated with Na¹³¹I and 5 μg thyroxine (Yamakazi et al. 1963).

TRH increases plasma TSH levels in thyroidectomized rats pretreated with 1 μg of T₃ (Bowers et al. 1965). This assay is based on enhanced TSH secretion after eliminating the feedback of thyroid hormones. The baseline is then lowered by T₃ pretreatment to provide a wider range for stimulation by TSH. Rats weighing 350–400 g are surgically thyroidectomized. After 1–3 months, 1 μg of L-triiodothyronine Na is given intraperitoneally; 2 h later urethane is given subcutaneously; approximately 1 h later 1.5 ml blood is removed from the jugular vein and the TRH preparation is administered intravenously. Another 1.5 ml blood is removed 15 min later. Heparin is added to the blood and TSH levels in plasma are assayed by release of ^{131}I from the mouse thyroid (McKenzie 1958) (see N.7.5.3). Results are recorded as mean change in blood ^{131}I levels in five mice.

TRH produces a significant depletion of pituitary TSH content in mice (Bowers et al. 1967). Swiss-Webster strain mice are treated as described for de-

N.9.1.3

Release of ^{131}I from Thyroid Glands of Mice

PURPOSE AND RATIONALE

An animal bioassay method for TRH utilizes iodine-deficient mice, treated with ^{131}I , codeine, and 1 μg thyroxine (Redding et al. 1966; Bowers et al. 1967; Red-

termination of blood ^{131}I radioactivity. Two hours after administration of TRF, the animals are decapitated and the pituitaries immediately removed. Pituitaries of each group of five mice are combined and homogenized in 5 ml of 0.01 M acetic acid which contains 0.9% NaCl. TSH is determined according to McKenzie (1958) (see N.7.5.3). Today, this has been replaced by radioimmunoassay determination of pituitary TSH content.

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- releasing factor (CRF) activity, has been modified to measure TRH activity *in vitro* (Guillemin et al. 1963; Bowers et al. 1965; Schally and Redding 1967).

PROCEDURE

Male Sprague-Dawley rats weighing 150–200 g serve as donors. After removal, each pituitary is cut in half, transferred to a 15-ml beaker containing 1.5 ml Krebs-Ringer bicarbonate medium with 200 mg% glucose and incubated for three 60-min periods. The media used in the first two incubations are discarded. At the beginning of the third incubation period, various amounts of test preparation or TRH standard are added to individual beakers. At the end of the third incubation period the media from both control and experimental beakers are carefully freed of pituitary tissue. The media are then assayed by RIA for content of TSH.

EVALUATION

Dose–response curves are established for test preparation and standard allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

For the assay of TRH analogs, cultures of enzymatically dispersed anterior pituitary cells from rats can be used instead of pituitary halves (Vale et al. 1972).

Barros et al. (1986) studied the effect of TRH on cultured GH3 rat anterior pituitary cells using the whole-cell voltage-clamp technique.

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N.9.1.4

Release of TSH from Rat Anterior Pituitary Glands *In Vitro*

PURPOSE AND RATIONALE

The *in vitro* bioassay method of Saffran and Schally (1955a, 1955b), developed for detecting corticotropin-

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N.9.1.5

TSH and Prolactin Release by TRH in Rats

In bioassays for TRH activity based on the release of TSH measured by radioimmunoassay it was found in many species investigated, and also in clinical testing of humans, that the injection of TRH causes the dose-proportional release of prolactin. This test is suitable for biological activity of TRH, e. g., in domestic animals, and also for the clinical investigation of prolactin secretion in humans.

PROCEDURE

The TRH test in rats is performed in a minimally invasive manner, and when animals have been previously treated it is done 24 h after the last dosing on the following morning. The test dose is a subcutaneous injection of 1 µg/kg body weight synthetic TRH followed by blood sampling in unanesthetized rats 30 min later. Blood sampling may be performed by retro-orbital puncture.

For extended studies on pituitary responsiveness, it is also possible to use a very specific TRH test blocking basal TSH by injection of levothyronine (L-T₃, 1 µg/rat s.c.) 18 h before the test. The following morning, the TRH dose is injected intravenously followed by blood sampling at 15 min or (in anesthetized rats) after 15 and 30 min.

EVALUATION

The usual procedure for evaluation is to compare the TSH concentration in control animals and treated animals (drug treatment or physiological manipulation), to calculate group means, and to evaluate the significance of difference by appropriate statistical tests, where necessary by performing analysis of variance (ANOVA).

The individual response of the animals may be assessed by comparing the basal TSH concentration be-

fore the TRH test injection, and the TSH increments at increasing dose intervals after TRH stimulation.

MODIFICATIONS OF THE METHOD

The TRH test is useful in rats and in other animal species. The test dose may be administered by intravenous injection or preferably by subcutaneous injection; nasal application should also be considered (a considerably higher TRH dose is required, and this test can be performed in dogs).

Several analytical methods are available to determine the serum concentrations of TSH and prolactin, both from commercial suppliers and for research applications (Fraglia 1998; Markianos et al. 1996; Katznelson 1998; Fail et al. 1999; Hashizume et al. 2005; Koch et al. 2006). For human diagnostic applications, highly sensitive TSH tests based on measuring only the basal concentrations have replaced the diagnostic TRH test in many instances. Methods are increasingly sensitive, and, for a review, refer to the web site "thyroidmanager" (Spencer 2004).

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N.9.2

Luteinizing Hormone Releasing Hormone (LH-RH)

N.9.2.1

General Considerations

Gonadotropin secretion is controlled by the hypothalamic peptide luteinizing hormone releasing hormone (LH-RH), which stimulates the release of FSH and LH.

The discovery of the structure of LH-RH then led to the synonym of gonadotropin releasing hormone (GnRH) and identification of its receptors followed (Conn et al. 1986). The search for a specific FSH-releasing factor has continued (McCann et al. 1993) but so far has remained elusive. LH-RH is secreted in a pulsatile fashion (Levine et al. 1991). This is not relevant to the bioassays, which use LH-RH injections as well as infusion for several hours. Radiolabeling and photoaffinity labeling of GnRH receptors have been described (Perrin et al. 1982; Hazum and Keinan 1983). LH-RH bioactivity is determined *in vitro* on pituitary cells and *in vivo* by its effects on ovulation, spermatogenesis, and other gonadal parameters (McCann 1970; Steelman 1970). Radioimmunoassays and radioreceptor assays are available (Nett and Niswender 1979). Many studies of rats and primates showed that prolonged administration of LH-RH agonists results in a decrease of LH and gonadal hormones to castrate levels (Sandow et al. 1978, 1980; Akhtar et al. 1983; Weinbauer et al. 1987, 1990).

Short- and long-term effects on pituitary-gonadal function in neonatal and adult female rats treated with gonadotropin-releasing hormones have been analyzed by Trimino et al. (1993).

The Food and Drug Administration (FDA) recommendations for preclinical testing of GnRH analogs, including pharmacology, pharmacokinetics and toxicology, are published by Raheja and Jordan (1994).

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N.9.2.2

LH-RH Receptor Assays

PURPOSE AND RATIONALE

LH-RH (GnRH) interacts with a membrane receptor which belongs to the G-protein-coupled receptor family. The LH-RH-R is encoded by a single-copy gene consisting of three exons and two introns. Consistent with its site of action, LH-RH-R mRNA has been found in the brain, pituitary, gonads, placenta, as well as in tumor tissue and tumor cell lines (Jennes and Conn 1994). Binding to LH-RH receptors in rat pituitary membranes has been studied for LR-RH agonists and antagonists. Furthermore, the time course of downregulation of LH-RH receptors was followed (Halmos et al. 1996).

PROCEDURE

Receptor binding of LH-RH is determined using a sensitive *in vitro* ligand competition assay based on binding of radiolabeled buserelin or [D-Trp⁶]LH-RH to rat anterior pituitary membrane homogenates (Halmos et al. 1993, 1996; Szöke et al. 1994). Membrane homogenates containing 40–80 µg of protein are incubated in triplicate with 60,000–75,000 cpm

(≈ 0.15 nM) [^{125}I][D-Trp⁶]LH-RH as radioligand and with increasing concentrations (10^{-12} – 10^{-6} M) of nonradioactive peptides in a total volume of 150 μl of binding buffer. At the end of the incubations, 125- μl aliquots of suspension are transferred to the top of 1 ml of ice-cold binding buffer containing 1.5% bovine serum albumin in siliconized polypropylene microcentrifuge tubes (Sigma). The tubes are centrifuged at 12,000g for 3 min at 4°C. Supernatants are aspirated, and the bottoms of the tubes containing the pellet are cut off and assayed in a gamma-counter. Protein concentration is determined by the method of Bradford (1976) using a BioRad protein assay kit.

EVALUATION

Specific ligand-binding capacities and affinities are calculated by the computerized curve-fitting program of Munson and Rodbard (1980) as modified by McPherson (1985). To determine the types of receptor binding, dissociation constants (K_d values), and the maximal binding capacity (B_{max}), LH-RH binding data are also analyzed by the Scatchard method. Statistical significance is assessed by Duncan's new multiple range test.

MODIFICATIONS OF THE METHOD

Flanagan et al. (1998) recommended ^{125}I -[His⁵,Tyr⁶]-Gn-RH as the radioligand for analysis of mutant Gn-RH receptors.

Perrin et al. (1982) compared binding of radiolabeled antagonists and agonists of gonadotropin-releasing hormone to rat anterior pituitary membrane homogenates.

Fekete et al. (1989) reviewed the role of receptors for LH-RH, somatostatin, prolactin, and epidermal growth factor in rat and human prostate cancers and in benign prostate hyperplasia.

Cloning, sequencing, and expression of the human gonadotropin-releasing hormone receptor are published by Kakar et al. (1992).

Marheineke et al. (1998) characterized the human gonadotropin-releasing hormone receptor heterologously produced using the baculovirus/insect cell and the semliki forest virus systems.

The binding kinetics of a long-acting gonadotropin-releasing hormone antagonist to rat LH-RH receptors were studied by Li et al. (1994).

Lovejoy et al. (1995) determined the receptor binding of gonadotropin-releasing hormone analogs in bovine pituitary membrane preparations.

The cDNA encoding the receptor for LH-RH was isolated from a human pituitary cDNA library and het-

erologously expressed in the murine fibroblast cell line LTK⁻ by Beckers et al. (1995).

Tsutsumi et al. (1995) investigated the role of altered receptor biosynthesis in agonist-induced receptor downregulation in αT_3 -1 cells, a mouse gonadotrope cell line.

Beckers et al. (1997) characterized gonadotropin-releasing hormone analogs by a cellular luciferase reporter gene assay. The assay is based on a fusion of the c-fos immediate-early gene promoter to *Photinus pyralis* luciferase (LUC) as reporter gene, stably transfected in murine LTK⁻ cells expressing the human GnRH receptor. Transcription of endogenous c-fos and fos-Luc fusion gene are transiently induced quite similarly by fetal calf serum and a superagonistic Gn-RH analog. The reporter gene was used to monitor agonist-induced signaling via the human Gn-RH receptor. Whereas Luc activity was induced in a dose-dependent manner by Gn-RH or an agonistic analog, different antagonistic peptides completely inhibited this stimulation.

The current state of knowledge is reviewed by Cheng and Leung (2005), Kakar et al. (2004) and Millar et al. (2004) for domestic animals and for the human, including the description of two separate types of the gonadotropin-releasing hormone receptor (Gn-RH-I and II), and the increasing complexity of Gn-RH receptor-mediated signaling.

CRITICAL ASSESSMENT OF THE METHODS

There is an ever-increasing complexity of the extensive results of the application of LH-RH (Gn-RH) receptor methods, which may be to structure-activity studies with LHRH analogs, to elucidating species-specific regulation in domestic animals and in humans, or to the measurement of adaptive changes of the LH-RH receptor concentrations in physiology, in pharmacological experiments, and in the pathophysiology of human disease. Numerous approaches have been described based on ligand binding to receptors extracted from tissue, to cloned receptor proteins, and based on direct measurement of the receptor protein concentrations.

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N.9.2.3

LH Release in the Ovariectomized Estrogen-Progesterone-Blocked Rat

PURPOSE AND RATIONALE

Ramirez and McCann (1963) recommended the ovariectomized, estrogen-progesterone-blocked rat (OEP rat) as a highly sensitive test model for LH-releasing activity. The gonadotropin content of the pituitary is increased after ovariectomy, due to reduced feedback inhibition through the absence of gonadal steroids. Basal secretion is then acutely lowered to a stable baseline by estrogen-progesterone blockade, whereas the amplitude for stimulation of LH secretion is augmented. The increase in plasma LH levels in donor rats after injection of LH-RH previously determined by bioassay in recipient rats is now conveniently measured by RIA. Since natural and synthetic LH-RH releases both gonadotropins (Schally et al. 1971b), merely stimulation of LH is necessary for routine LH-RH assay.

PROCEDURE

Female Sprague-Dawley rats weighing 150–200 are ovariectomized. They are kept in a light- and temperature-controlled animal room at 24°C and are fed commercial rat chow and tap water ad libitum. Tests are performed 1–3 months after ovariectomy. Three days prior to the assay, the rats receive 50 µg estradiolbenzoate and 25 mg progesterone in sesame oil by the transmuscular-subcutaneous route. For the assay, the rats are anesthetized by subcutaneous injection of 0.6 ml/100 g of 25% urethane solution. Changing to a new anesthetic will require validation of the effect of that anesthetic on sensitivity of the test. Various doses of the test compound or the standard

are injected intravenously into the jugular vein. After 10 min, 4–6 ml blood is withdrawn by cardiac puncture from each donor allowing the separation of 2 ml plasma.

In the original method, bioassayable LH was measured by the ovarian ascorbic acid depletion (OAAD) method according to Parlow and Reichert (1963) (see also N.7.1.2.3). Immature female Sprague-Dawley rats are injected on day 1 at 3:00 p.m. with 50 IU pregnant mares' serum gonadotropin (PMSG) in 0.2 ml saline subcutaneously. On day 3, the rats receive at 9:00 a.m. 25 IU human chorionic gonadotropin (hCG) subcutaneously. On day 7, three different doses of the standard (e. g., NIH LH-S-1, National Institute of Health, Bethesda, Md., USA) or the 2 ml of plasma from the OEP rats is injected intravenously. Eight animals are used per group. Three hours later, the animals are sacrificed, both ovaries are prepared, weighed, and homogenized for determination of ascorbic acid content. The LH activity in OEP plasma, as measured by OAAD, is expressed in terms of NIH-LH-S16 standard.

Furthermore, LH activity in the plasma of OEP rats is measured by radioimmunoassay. OEP rats are anesthetized with 25% urethane intraperitoneally. Blood is withdrawn by retro-orbital puncture. Then various doses of the test preparation or the standard are injected intravenously. After 10 min blood is withdrawn by cardiac puncture. Radioimmunoassays of LH are carried out by the double-antibody method of Niswender et al. (1968).

EVALUATION

Dose–response curves are established for the standard and the test preparation of LH-RH measured by bioassayable LH in OEP plasma as well as for RIA of the LH level in plasma, allowing the calculation of potency ratios with confidence limits. Furthermore, time–response curves can be established by RIA determination of plasma LH levels in OEP rats.

MODIFICATIONS OF THE METHOD

Instead of OEP rats, normal Male Sprague-Dawley rats can be used for measurement of the time course of release of LH after injection of LH-RH or LH-RH derivatives (Arimura et al. 1972). Male Sprague-Dawley rats weighing 120–150 g are anesthetized with 0.6 ml/100 g body weight of 25% urethane solution subcutaneously. After 30 min 0.8 ml blood is withdrawn from the jugular vein, and immediately substituted by the same volume of Haemacel solution. The LH-RH preparation is injected sub-

cutaneously in 1% gelatine-saline. Blood is withdrawn at hourly intervals up to 6 h, each time being substituted with Haemacel solution. LH in plasma is determined by the double-antibody method of Niswender et al. (1968) and follicle-stimulating hormone according to the method of Daane and Parlow (1971).

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N.9.2.4**Gonadotropin Release From Anterior Pituitary Cells****PURPOSE AND RATIONALE**

Anterior pituitaries can be used directly, kept in culture or used for cell lines (Mittler and Meites 1964, 1966; Mittler et al. 1970; Sandow et al. 1972b) in order to study the synthesis and release of gonadotropins in response to LH-RH.

PROCEDURE

Female Sprague-Dawley rats weighing 100–150 g are used as donors. Each anterior pituitary is removed and cut into four to six pieces of approximately equal size. The cultures are performed in sterile disposable plastic Petri dishes each containing 3 ml medium consisting of 9 parts Difco medium 199 and 1 part of newborn calf serum; 25 U/ml penicillin and 25 µg/ml streptomycin are added. In each dish, the explants are supported at the gas interface. An atmosphere of 95% oxygen and 5% carbon dioxide and a temperature of 36°C are maintained. The opposite sides of the same pituitaries provide matched control and experimental preparations. The pituitaries are incubated for a total time of 5 days. After the first 2 days, the medium is removed and discarded. Fresh medium is then added with the LH-RH solutions. Approximately 12 h after the first change of medium and addition of LH-RH, media are removed and frozen. Fresh medium with LH-RH is again added; this procedure is repeated until six samples of medium representing the last 3 days of culture are obtained. Media are assayed for LH content by radioimmunoassay according to Niswender et al. (1968) and for FSH content according to Parlow and Reichert (1963).

EVALUATION

Using various concentrations of test preparation and LH-RH standard dose–response curves are obtained allowing the calculation of potency ratios with confidence limits.

CRITICAL ASSESSMENT OF THE METHOD

These methods do not reflect the time course of release found *in vivo* but are useful for potency estimates.

MODIFICATIONS OF THE METHOD

Instead of pituitary halves for the assay of LH-RH as well as for the assay of TRF and its analogs, cultures of enzymatically dispersed anterior pituitary cells from rats can be used (Vale et al. 1972; Martin and Sattler 1979).

Loughlin et al. (1981) used perfused pituitary cultures as a model for LH-RH regulation of LH secretion.

O'Connor and Lapp (1984) studied the effect of pulse frequency and duration of exposure to LH-RH in anterior pituitary cells attached to Cytodex I beads.

The functional integrity of anterior pituitary cells separated by a density gradient has been studied (Scheikl-Lenz et al. 1985).

The receptor binding ability of different agonists and antagonists of LH-RH to rat pituitary and human breast cancer membranes was studied by Fekete et al. (1989).

Vigh and Schally (1984) and Csernus and Schally (1991) described in detail a cell superfusion system consisting of a Sephadex column with dispersed pituitary cells. The LH response of anterior pituitary cells to a 3-min exposure to various concentrations of LH-RH at 30-min intervals as well as the growth hormone (GH) response to human GH-releasing hormone resulted in excellent dose–response curves. The effect of GH-releasing hormone was inhibited by somatostatin. Likewise, the effect of LH-RH was inhibited by pretreatment with LH-RH antagonists.

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N.9.2.5

Radioimmunoassay of Rat LH

PURPOSE AND RATIONALE

Luteinizing hormone is a glycoprotein hormone exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin-releasing hormone activity, a homologous assay is necessary. The reagents are provided by the National Pituitary Agency, Bethesda, Md. The assay procedure is similar to the standard operating procedure proposed by the National Pituitary Agency, USA.

PROCEDURE

Reagents

| | |
|------------------|-------------------------------------------------------------------------|
| Standard: | NIH-rat-LH-RP 1 |
| Antiserum: | rabbit-anti-rat-S 9 |
| Tracer: | ¹²⁵ I-rat-LH-I 6 |
| Second antibody: | Behring goat-anti-rabbit-gamma-globulin, (Behring, Cat. No. OTOP 14/15) |
| Buffer: | 0.01 M phosphate-saline pH 7.4 |

Standard and samples are dissolved in 1% bovine serum albumin, tracer in 0.1% bovine serum albumin, and antiserum in EDTA-PBS (1:350 normal rabbit serum as carrier).

Assay

| | |
|------------|-------------------------------------------------------|
| Standards: | 0.25–62.5 ng/tube, 200 µl/tube |
| Antiserum: | 1:20,000 100 µl/tube |
| Tracer: | specific activity 120 µCi/µg, 8000 cpm in 100 µl/tube |

Standards (or sample) are incubated with antiserum and tracer for 48 h at +4°C, the second antibody 1:50 (200 µl/tube) is added, and incubated for 24 h at +4°C. Separation is performed with 1.0 ml ice-cold phosphate-buffered saline pH 7.4, the vial spun at 1300 g for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma-counter.

EVALUATION

Data processing: standard curves and sample data are calculated on a computer program using a spline function.

Quality control parameters

| | |
|-------------------------------------------------------------------------------------------------|-----------------------------|
| Limit of detection: | 0.36 ng per tube |
| Standard curve (<i>ED</i> ₈₀ – <i>ED</i> ₅₀ – <i>ED</i> ₂₀): | 1.31–4.56–15.74 ng per tube |
| Inter-assay CV (15 assays): | 20.9% |
| Intra-assay CV: | lt; 15% |

REFERENCES AND FURTHER READING

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N.9.2.6

Radioimmunoassay of Rat Follicle-Stimulating Hormone (FSH)

PURPOSE AND RATIONALE

Follicle-stimulating hormone (FSH) is a glycoprotein hormone exhibiting species specificity. For pharmaco-

logical experiments in rats, such as evaluation of gonadotropin-releasing hormone activity, a homologous assay is necessary. The reagents and the procedure are provided by the National Pituitary Agency, Bethesda, Md. The assay procedure is similar to the standard operating procedure proposed by the National Pituitary Agency, USA.

PROCEDURE

Reagents

| | |
|------------------|-------------------------------------------------------------------------|
| Standard: | NIAMDD-rat-FSH-RP-1 |
| Antiserum: | rabbit-anti-rat-FSH (NIAMDD-S-9) |
| Tracer: | ¹²⁵ I-rat-FSH (e. g. NIAMDD-I-4) |
| Second antibody: | Behring goat-anti-rabbit-gamma-globulin, (Behring, Cat. No. OTOP 14/15) |
| Buffer: | 0.01 M-phosphate-saline/0.1% bovine serum albumin, pH 7.4 |

Assay

| | |
|------------|---------------------------------------------------------|
| Standards: | 6.25–1600 ng/tube, 200 µl/tube |
| Antiserum: | 1:2000 100 µl/tube |
| Tracer: | specific activity 200 µCi/µg, 10,000 cpm in 100 µl/tube |

Standards (or sample) are incubated with antiserum and tracer for 72 h at +4°C, the second antibody 1:50, 200 µl/tube, is added, and incubated for 48 h at +4°C. Separation is performed with 1.0 ml ice-cold phosphate-buffered saline pH 7.4, the vial spun at 1300 g for 15 min, the supernatant decanted, and the residue counted for 1 min in a gamma-counter.

EVALUATION

Data processing: standard curves and sample data are calculated on a computer program using a spline function.

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N.9.2.7

Measurement of Ascorbic Acid Depletion in Ovaries of Pseudopregnant Rats

PURPOSE AND RATIONALE

The assay for LH-RH and LH-RH analogs can be performed in one step in pseudopregnant immature

female rats using the biological response of the luteinized ovaries to gonadotropins released by the test compounds. Pseudopregnancy is induced in immature female Sprague-Dawley rats by treatment with gonadotropins. Numerous corpora lutea are formed after ovulation. On days 7–8 after the start of treatment the corpora lutea are very sensitive to endogenous or exogenous gonadotropins. Stimulation of steroid synthesis, mainly progesterone, is seen, associated with dose-dependent ascorbic acid depletion in the ovaries. The activity of LH-RH and LH-RH analogs can be determined by the decrease of ovarian ascorbic acid concentration, or the increase in progesterone secretion. Ascorbic acid depletion is a sensitive parameter for the endogenous gonadotropin release in the animals.

PROCEDURE

Immature female Sprague-Dawley rats weighing 35–45 g are pretreated with 50 IU pregnant mares' serum gonadotropin (PMSG) followed by an injection of 25 IU human chorionic gonadotropin (hCG) on the third day. On day 7 or 8, they are injected intramuscularly or subcutaneously with the test preparation or the LH-RH standard in 0.1 ml 1% gelatine-saline. Eight animals are used for each of three doses of the test preparation and standard. One hour later, the ovaries are dissected out, homogenized, and ascorbic acid is determined according to the method of Mindlin and Butler (1938) by photometry.

EVALUATION

Dose–response curves of ascorbic acid depletion are obtained for the test preparation and standard allowing the calculation of potency ratios with confidence limits.

MODIFICATION OF THE METHOD

The method has been adopted as a biological assay of gonadorelin with some modifications by pharmacopoeias, e. g., the British Pharmacopoeia (1988).

CRITICAL ASSESSMENT OF THE METHOD

Classical bioassays of the kind described here are now rarely applied for standardizing LH-RH (gonadorelin) due to the complexity of the biological method. They are being replaced by analytical methods of high specificity and sensitivity, e. g., high-performance liquid chromatography (HPLC) which has been compared with the biological assay and found to be suitable for standardization (see current versions of the British Pharmacopoeia). Standard preparations for gonadorelin (LH-RH, Gn-RH) and for LH-RH analogs

may be obtained from the suppliers (pharmaceutical companies); they are no longer prepared by the National Institute for Biological Standards and Control (NIBS, UK).

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N.9.2.8

Progesterone Production in Pseudopregnant Rats

PURPOSE AND RATIONALE

Pseudopregnancy is induced in immature rats by pre-treatment with gonadotropins. Numerous corpora lutea are formed after ovulation that are sensitive to endogenous gonadotropins released by LH-RH or LH-RH analogs on days 6–8 after treatment. By measurement of plasma progesterone, the steroidogenic activity of LH-RH analogs can be determined (Sandow et al. 1976).

PROCEDURE

Immature female Sprague-Dawley rats weighing 35–45 g are pretreated with 50 IU pregnant mares' serum gonadotropin (PMSG) followed by an injection of 25 IU human chorionic gonadotropin (hCG) on the third day. They are injected intramuscularly with LH-RH or the LH-RH analog on days 6, 7, or 9 between 8 and 10 a.m. Eight animals are used for each of three doses of test preparation and standard. Blood samples are collected 1 h after treatment. Plasma samples of equal volume are extracted with peroxide free diethylether. The ether phase containing progesterone is evaporated and the sample re-dissolved in BSA-phosphate buffer. Tritium-labeled progesterone and a spe-

cific antiserum against progesterone are added and incubated over a period of 24 h at 4°C. Bound hormone and free hormone are separated by absorption on Dextran-coated charcoal. The activity of the sample is determined in a scintillation cocktail containing Triton-X.

EVALUATION

Dose–response curves for progesterone concentrations are established for the test preparation and standard, allowing the calculation of potency ratios with confidence limits.

REFERENCES AND FURTHER READING

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N.9.2.9

Induction of Ovulation in Rabbits

PURPOSE AND RATIONALE

Ovulation can be induced in mature rabbits by injection of LH-RH after initial priming. This is a modified assay for LH release, which was applied to hypothalamic extracts containing LH-RH activity (Sandow et al. 1972).

PROCEDURE

Six unmated, mature female rabbits (Himalayan strain) weighing 1.0–1.2 kg are used for each dose of standard or test preparation. Follicular maturation is induced by eight subsequent daily subcutaneous injections of LH-RH or the LH-RH analog to be tested in 0.2 ml 1% gelatine-saline. Then 48 h after the last injection, ovulation is induced by administering a fourfold to tenfold higher dose of the peptide in 0.2 ml subcutaneously. After 24 h, the animals are sacrificed, the ovaries weighed, and ovulation is determined by counting the numbers of follicles ovulated in the ovaries, as defined by local bleeding.

EVALUATION

At least two doses of test preparation and standard are used in order to establish dose–response curves, allowing the calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Schröder et al. (1972) and Sandow and Hahn (1973) described changes in the ovary transplanted to the anterior eye chamber in the rabbit enabling the direct ob-

ervation of ovulation. This method is also applicable to steroids and prostaglandins.

CRITICAL ASSESSMENT OF THE METHODS

Classical assays such as the eye-ovary transplant have provided excellent morphological information about changes of the ovary during drug treatment. They are however becoming increasingly obsolete because of other methods which are much less invasive, and their use should be considered only as an exception.

REFERENCES AND FURTHER READING

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N.9.2.10

Induction of Superovulation in Immature Rats

PURPOSE AND RATIONALE

Immature female rats (24–26 days of age) do not show vaginal cycles before the onset of puberty. Treatment with pregnant mares' serum gonadotropin (PMSG) induces follicular maturation, followed by spontaneous ovulation 2 days later. Spontaneous ovulation can be blocked by barbiturates (e. g., phenobarbital) acting on the hypothalamus, and is overcome by exogenous LH-RH. The rats ovulate due to the release of endogenous gonadotropins. After hypophysectomy, no ovulatory activity is observed in the absence of the pituitary (Sandow et al. 1972).

PROCEDURE

Immature female Sprague-Dawley rats weighing 55–65 g are injected on day 1 with 10 IU PMSG subcutaneously. Following this pretreatment, the animals will ovulate spontaneously on day 3 between 2:00 and 4:00 p.m. Spontaneous ovulation is blocked by phenobarbital 4 mg/kg at 1:00 p.m. One hour later, the ovulatory peptide, dissolved in 1% gelatine/saline, is injected intravenously. Controls are treated with 1 or 2 IU human chorionic gonadotropin (hCG); negative controls, with gelatine-saline only. Eight rats are used for control groups and various doses of LH-RH or the LH-RH analog. On the next day, the rats are sacrificed at 11:00 a.m. and the oviducts are dissected and stained

with Patent blue. The number of ova is counted under a microscope.

EVALUATION

A dose-dependent increase in the number of ova per rat is observed after LH-RH due to LH release and after hCG by direct action on the ovary. The effect of LH-RH analogs is compared with the effects of hCG. Minimal effective doses for LH release can be calculated.

REFERENCES AND FURTHER READING

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N.9.2.11

Inhibition of Experimentally Induced Endometriosis

PURPOSE AND RATIONALE

This is an example of a disease model in animals designed to mimic treatment of an experimentally induced pathological condition. Endometriosis-like lesions can be induced in female rats by autotransplantation of endometrium under the renal capsule (Sakata et al. 1990; Mizutani et al. 1995). This method was used to compare the effect of steroid-induced suppression by a LH-RH analog after chronic administration (pituitary inhibition).

PROCEDURE

Under anesthesia laparotomy is performed in 9-week-old female Sprague-Dawley rats. The left uterine horn is resected and opened by a longitudinal incision. The endometrium is dissected from the myometrium. Then a 5 × 5 mm section of the endometrium is grafted under the capsule of the left kidney of the same animal. Two weeks later, the attachment and the viability of the endometrial explant are examined by a second laparotomy, the length, width, and height of the explant are measured, and the volume is then calculated. The criterion for a viable graft is fluid accumulation around the lesion. The rate of induction of endometriosis is more than 80%. Animals with endometriosis (with body weights of approximately 250 g) are randomly divided into treatment groups of ten animals each. Gonadotropin-releasing hormone agonists are injected at doses of 15 or 30 µg/kg subcutaneously daily for 3 weeks. A third laparotomy is performed 3 weeks after the beginning of the experiment. The presence of

fluid accumulation and the size of the endometrial explant are examined. The explant is excised and fixed in 10% formalin for histological evaluation.

EVALUATION

The evaluation of treatment on the regression of lesions in experimental endometriosis is analyzed statistically with the χ^2 test.

REFERENCES AND FURTHER READING

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N.9.3

LH-RH Antagonistic Activity

N.9.3.1

Testosterone Suppression in Rats

PURPOSE AND RATIONALE

LH-RH antagonists suppress endogenous secretion of LH and testosterone in adult male rats after a single subcutaneous injection, and after repeated dosing (Loy 1994).

PROCEDURE

Groups of eight adult male Wistar rats weighing 200–250 g are injected subcutaneously with various doses of the test compound or a reference LH-RH antagonist dissolved in 5% mannitol solution. Four hours later, rats are decapitated and blood is collected from the trunk. Serum is separated in a refrigerated centrifuge (10 min at 3000 g), and stored frozen at -20°C until assay.

Radioimmunoassay for Testosterone

Serum testosterone is measured by radioimmunoassay in serum extracts using a specific antiserum without prior chromatography, or by direct assay in serum depending on the method. Serum samples of 0.5 ml are extracted with 2 ml of freshly purified, peroxide-free diethylether by shaking for 60 s on a Vortex-type mixer. The aqueous phase is frozen at -70°C , and the ether phase containing testosterone is transferred to conical test tubes, and evaporated under a stream of dry nitrogen. The dry residue is re-dissolved in

BSA/phosphate buffer (BSA = 1% bovine serum albumin) for RIA. $[1,2,6,7-^3\text{H}]$ Testosterone (New England Nuclear NET 367) and a specific antiserum (AS-781, Behringwerke, Marburg, Germany) are added and incubated over a period of 24 h at $+4^{\circ}\text{C}$ under nonequilibrium conditions. Bound hormone and free hormone are separated by adsorption on Dextran-coated charcoal by incubation for 30 min at $+4^{\circ}\text{C}$ and centrifugation at 3000 g for 15 min. Then 500 μl of supernatant is transferred into minivials and scintillation cocktail is added. Radioactivity is determined in a beta-counter (Falvo and Nalbandov 1974).

EVALUATION

The hormone levels in the sample are calculated from a standard curve by means of a computer program, using appropriate control sera. Using various doses of standard and test preparation dose–response curves can be established, allowing calculation of potency ratios with confidence limits. Alternatively, minimum effective doses can be calculated from comparisons with controls. Using different time intervals, e. g., 4, 8, and 24 h, the duration of the effect can be evaluated.

MODIFICATIONS OF THE METHOD

Testosterone suppression can be measured in several animal species, e. g., dogs and marmoset monkeys, or cynomolgus monkeys (Habenicht et al. 1990).

Ayalon et al. (1993) tested the potency of the LH-RH antagonist on the pituitary–gonadal system of female castrated and intact ovulating rats.

Reissmann et al. (1996) investigated the anti-tumor and hormone-suppressive effect of the LH-RH antagonist cetrorelix in the model of DMBA-induced mammary carcinoma (DMBA is 7,12-dimethylbenz[a]anthracene) in female rats and by testosterone determination in normal male rats.

Danz (1995) studied the effect of a gonadotropin-releasing hormone antagonist on androgen-binding protein production and its distribution among the epididymis, seminiferous tubule fluid, testicular interstitial fluid, and blood in rats.

Fallest et al. (1995) studied the transcriptional regulation of the rat luteinizing hormone β (rLH β) gene through the use of **transgenic mice** bearing a region of the rLH β gene linked to a luciferase (LUC) reporter gene. The postgonadectomy rise in pituitary rLH β LUC activity in females and males was blocked by daily administration of the Gn-RH antagonist antide.

Rivier et al. (1996) synthesized and evaluated many Gn-RH analogs and established a dose–response re-

lationship between Gn-RH antagonists and pituitary suppression.

CRITICAL ASSESSMENT OF THE METHOD

Structure–activity studies on LH-RH antagonists are based on screening for receptor affinity, and receptor-associated signaling for high-throughput assays. They need to be followed up by confirmation of biological activity, and for quantitation of structure–activity assessment, the assays described here have been eminently suitable for extended compound screening, although the estimates obtained need to be checked for their relevance against the results from intact animals. Suppression of testosterone secretion is one of the preferred methods of confirmation.

REFERENCES AND FURTHER READING

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- blocked by barbiturates (e.g., phenobarbital) to prevent spontaneous ovulation (see Sect. N.9.2.10). For antioviulatory activity, a test dose of 800 ng exogenous LH-RH is administered together with increasing doses of the LH-RH antagonist. The antagonist will then inhibit the LHRH-induced gonadotropin release. The test measures the dose of LH-RH antagonist required to inhibit the effect of a standard dose of LH-RH.

PROCEDURE

Immature female Wistar rats weighing 55–65 g are injected on day 1 at 9:00 a.m. with 10 IU PMSG subcutaneously. This priming induces follicular maturation and estradiol secretion. A spontaneous, endogenous LH discharge is observed on day 3 between 2:00 and 4:00 p.m.. The release of LH and spontaneous ovulation is blocked by intraperitoneal injection of phenobarbital 4 mg/kg on day 3 at 1:00 p.m.. The LH-RH antagonist is administered in various doses subcutaneously, intraperitoneally or intravenously 30 min before phenobarbital injection. Two hours later, a standard dose of 800 ng LH-RH is injected subcutaneously to induce ovulation. Control groups receive phenobarbital only (negative control) or phenobarbital and 800 ng LH-RH (positive control). Six to eight rats are used for control groups and various doses of the LH-RH antagonist. On the next day, the rats are sacrificed at 9:00 a.m. Both ovaries are prepared and weighed. The oviducts are dissected and stained with Patent blue. The number of ova is counted under a microscope. An effective dose of antagonist prevents LH-RH-induced ovulation.

EVALUATION

A dose-dependent suppression of induced ovulation is observed after the LH-RH antagonist. ID_{50} values can be calculated for various LH-RH antagonists according to the procedure of Litchfield-Wilcoxon. Minimal effective doses which fully suppress ovulation in each animal are calculated.

N.9.3.2

Antioviulatory Activity in Rats

PURPOSE AND RATIONALE

The rat antioviulatory assay (AOA) was widely used for LH-RH antagonists. Immature female rats (24–26 days of age) are primed with pregnant mares' serum gonadotropin (PMSG) to induce follicular maturation, followed by spontaneous ovulation 2 days later. The endogenous hypothalamic LH-RH discharge can be

MODIFICATIONS OF THE METHOD

De la Cruz et al. (1975) described the blockade of the preovulatory LH surge in **hamsters** by an inhibitory analog of LH-RH.

Kovács et al. (1993) found that in ovariectomized and normally cycling rats antioviulatory doses of antagonists of LH-RH inhibit LH and progesterone but not follicle-stimulating hormone (FSH) and estradiol release.

Evaluation of biological activities of new LH-RH antagonists in male and female rats was reported by Pinski et al. (1993).

Rivier et al. (1995) tested a series of gonadotropin-releasing hormone antagonists in the rat antiovarulatory assay.

Pinski et al. (1995) evaluated the optical isomers of the LH-RH antagonist Cetrorelix in ovulation inhibition in rats and in suppression of LH levels.

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N.9.3.3

Effect of Repeated Administration of LH-RH Antagonists in Rats

PURPOSE AND RATIONALE

Repeated administration of LH-RH antagonists reduces the testosterone secretion in serum and the tissue content in the testes, and it also decreases the weight of testosterone-dependent organs, such as prostate and seminal vesicles. Moreover, pituitary LH content is decreased and the secretory capacity of the testes for testosterone is diminished. The effects are similar to those after supraphysiological doses of LH-RH agonists (paradoxical antifertility effects).

PROCEDURE

Male Wistar rats with an initial weight between 150 and 200 g are housed under standard conditions. They are treated with daily injections of the LH-RH antagonist for a period of 7 days up to 4 weeks. Alternatively, the LH-RH antagonist can be administered subcutaneously by infusion via minipumps or other routes of administration. Animals receiving the vehicle serve as controls, and rats castrated at the beginning of the experiment may be included for maximum inhibition. At the end of the treatment period, the animals are sacrificed 4 h after the last administration. Blood is collected for testosterone determination by radioimmunoassay. The androgen-dependent organs, testes, epididymides, ventral prostate, and seminal vesicles are dissected and weighed to the nearest 0.1 mg. The testes are decapsulated and incubated with 250 mU human chorionic gonadotropin (hCG) in order to determine the secretory capacity for testosterone. The testosterone tissue content is determined in the supernatant fraction of a testicular homogenate in the absence of hCG (unstimulated testis), and after 3 h of incubation of the contralateral testis with hCG (stimulated testis). Pituitary glands are dissected and the anterior lobe is frozen at -20°C for determination of LH by rat-specific radioimmunoassay (Sect. N.9.2.5). LH receptors in anterior pituitary homogenate are measured by binding of ^{125}I -buserelin *in vitro*.

EVALUATION

The effects of various doses of the LH-RH antagonist after various time intervals on the different parameters mentioned above are compared with values from intact controls and castrated animals. Significant differences compared to controls at the 95% level are calculated by Dunnett's test.

MODIFICATIONS OF THE METHOD

These are mainly models for contraceptive applications and tumor suppression using LH-RH antagonists.

Kangasniemi et al. (1996) used a combined treatment with a Gn-RH antagonist and an antiandrogen (flutamide) to suppress spermatogenesis in mice. Despite this effect, the treatment did not enhance recovery from spermatogenesis produced by a 10-Gy dose of radiation.

Sinha-Hikim and Swerdloff (1993, 1994) examined the time course of suppression and recovery of spermatogenesis and its relationship to the temporal changes in circulating levels of gonadotropin and testosterone and intratesticular testosterone levels after cessation of treatment with a potent Gn-RH antagonist.

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N.9.3.4

Inhibition of Gonadotropin Release From Anterior Pituitary Cultures

PURPOSE AND RATIONALE

The same methods as described for studying the stimulation of gonadotropin release by LH-RH agonists (Sect. N.9.2.3, N.9.2.4) can be used for assessment of LH-RH antagonists.

PROCEDURE

Anterior pituitaries of young adult Sprague-Dawley rats are digested with collagenase for 1 h followed by a mechanical dispersion. The resulting cell suspension from 1.5 pituitaries, containing mostly small clusters of cells, is then sedimented together with a suspension of Sephadex G-10 (Sigma) and packed into 6.6-mm columns. Tissue culture medium 199 (Sigma) with supplements, equilibrated with 95% air/5% carbon dioxide, is perfused through the columns at a flow rate of 0.33 ml/min. After an overnight recovery period, during which the baseline stabilizes and the cells regain their full responsiveness, the samples to be tested are introduced through a four-way valve. During a 9-h experimental period, 180 1-ml fractions are collected. The system is standardized with 3-min exposures to 100 mM potassium chloride or 3 nM LH-RH. The compounds are introduced in various concentrations, generally for 3-9 min (time of one to three fractions), at 30-min intervals. Rat LH levels are measured from aliquots (50 µl) of the collected medium effluent by radioimmunoassay. As a standard, rat LH-RP2 reference standard is used. Repeated stimulation with 3 nM LH-RH for 3 min at 30-min intervals results in a pulsatile LH release.

EVALUATION

The suppression of pulsatile LH release due to repeated administration of LH-RH by antagonists is evaluated.

MODIFICATIONS OF THE METHOD

Krummen et al. (1991) assessed the direct effects of testosterone in primary cultures of pituitary cells.

A special long-term superfusion system was developed by Rékási et al. (1993) in order to evaluate the effects of cytotoxic compounds linked to LH-RH analogs on different types of rat pituitary cells. LH, growth hormone and prolactin were determined simultaneously in the effluent.

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N.9.3.5

Anti-Tumor Effect of LH-RH Antagonists

PURPOSE AND RATIONALE

LH-RH antagonists were found to inhibit experimental tumors in rats and mice.

PROCEDURE

To induce mammary carcinomas, female rats are each given a single dose of DMBA (dimethylbenzanthracene) at the age of 50 days. The first mammary tumors can be detected 20–30 days later. The tumor weight is determined by palpation, comparing the volume of each tumor to that of preformed Plasticine models. The tumor weight is calculated by multiplication of the model weight by a factor which takes account of the specific weights of Plasticine and tumor tissue. After the total tumor mass per animal has reached about 1 g, the animals are randomly divided into treatment groups. At least eight animals are used per group. The rats are treated for 3 weeks with different subcutaneous doses of LH-RH antagonists or ve-

hicle. The experiment is terminated at the end of the 3rd week from the initiation of the experiment. Histological examination of the tumors is performed.

EVALUATION

The change in tumor volume is calculated on the basis of individual responses. For determination of the mitotic index, 4000 cells are considered in each tumor.

MODIFICATIONS OF THE METHOD

Reduction of tumor weight in female BDF₁ mice bearing MXT mammary adenocarcinomas after treatment for 3 weeks with a LH-RH antagonist was reported by Szende et al. (1990).

Inhibition of MIA PaCa-2 human pancreatic xenografts in nude mice by a LH-RH antagonist was reported by Radulovic et al. (1993).

The involvement of insulin-like growth factors in growth regulation of the Ishikawa endometrial tumor cell line and the possible interference by LH-RH analogs was evaluated by Kleinman et al. (1993).

Pinski et al. (1994) investigated the effects of treatment with a LH-RH antagonist and a LH-RH agonist in Copenhagen rats bearing the anaplastic androgen-independent Dunning R-3327-AT-1 prostatic adenocarcinoma implanted orthotopically into the ventral lobes of prostate glands.

Vincze et al. (1994) tested the anti-tumor effect of the gonadotropin-releasing hormone antagonist MI-1544 *in vitro* on human breast cancer lines and *in vivo* on xenografts in immunosuppressed mice.

Manetta et al. (1995) reported the *in vitro* and *in vivo* inhibitory effects of a LH-RH antagonist against a panel of human ovarian carcinomas.

The effects of LH-RH and a LH-RH antagonist on cell growth and production of hCG and cAMP in JAR human chorioncarcinoma cells were examined *in vitro* by Horváth et al. (1995).

CRITICAL ASSESSMENT OF THE METHOD

In many instances, research is now based on assessing and confirming the anti-apoptotic activity of tumor-inhibiting compounds, e.g., methods based on thymidine incorporation to assess tissue proliferation may be considered. In the case of LH-RH antagonists the use of biological systems based on transplanted tumors in animals reflects the suppression of pituitary hormone release followed by reduced secretion of gonadal steroids. Testing the effect on experimental tumors in animals is a recommended procedure for obtaining confirmation of the clinical potential of application.

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N.9.4

Corticotropin-Releasing Hormone (CRH)

N.9.4.1

General Considerations

A hypothalamic factor inducing release of corticotropin (ACTH) from pituitaries was the first of the hypothalamic releasing hormones identified using *in vitro* bioassays (Saffran and Schally 1955; Guillemin and Rosenberg 1955). A common pre-pro-hormone, opiomelanocortin, was identified as the source of corticotropins and endorphins. Much later, the structure of a 41-residue ovine hypothalamic peptide stimulating the secretion of corticotropin and β -endorphin was identified (Vale et al. 1981), followed by elucidation of the structure of human CRH (Shibahara et al. 1983) and of other species, such as porcine corticotropin-releasing factor (CRF; Patthy et al. 1985) and equine CRF (Livesey et al. 1991). Reviews are given by Brodish (1979), Rivier and Plotsky (1986), and Taylor and Fishman (1988), Vigh et al. (1982), Koob (1999).

Conformational differences between ovine and human CRH were detected using circular dichroism, Fourier transform infrared spectroscopy, nuclear magnetic resonance (NMR) and dynamic light scattering (Dahte et al. 1996).

The binding sites of immunoreactive CRH in the rat ovary and its potential physiological role were studied by Mastorakos et al. (1993).

Studies on CRF receptors in the pituitary have been performed (Wynn et al. 1983; Millan et al. 1987). Investigations into the general pharmacological properties of human CRH did not reveal any considerable side-effects (Andoh et al. 1994).

Derivatives with agonistic and antagonistic properties have been synthesized and tested (Rivier et al. 1984; Kornreich et al. 1992; Chen et al. 1996; Schulz et al. 1996; Webster et al. 1996; Arai et al. 1998).

The role of CRH in inflammatory processes was investigated by Webster et al. (1998).

Non-mammalian peptides sauvagine (from frog) and **urotensin 1** (from fish) have approximately 50% sequence homology with CRF and share *in vitro* and physiological actions characteristic of CRF (Rivier et al. 1983). A mammalian urotensin-like peptide (named **urocortin**) with partial sequence identity with urotensin 1 and CRF has been identified in rat (Vaughn et al. 1995) and human (Donaldson et al. 1995) tissues. The mouse and human urocortin genes have been isolated and characterized (Zhao et al. 1998).

A **corticotropin-releasing factor binding protein** (CRFBP) was isolated and is thought to be an important modulator of CRF in both the CNS and the periphery (Behan et al. 1995; Petraglia et al. 1996; Cortright et al. 1997; Hobel et al. 1999). CRFBP levels rise at 30–35 weeks of pregnancy and dramatically decrease at 38–40 weeks. *In vitro*, CRFBP inhibits the ACTH-releasing properties of CRF.

Cortright et al. (1995) described a mouse brain CRH-binding protein (CRHBP) highly homologous to human and rat CRHBPs, but distinct from the CRH receptor.

Stenzel-Poore et al. (1996) described **transgenic mice** with CRH overproduction that induces behavioral changes that parallel the stress syndrome. Van Gaalen et al. (2002, 2003) reported anxiety-like behavior and reduced attention in mice overproducing CRH in an operant five-choice serial task, which taxes sustained and divided attention.

Bale et al. (2002) found that mice deficient for both CRF receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior.

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N.9.4.1.1

Central Effects and Actions of CRH

In some contrast to the initial conception of a hypothalamic factor regulating adrenocortical function, research on CRH, related CRF peptides and nonpeptidic compounds interacting with the CRH receptors, has established for CRH both a central role and important peripheral effects. In keeping with knowledge about the effects of neurohypophyseal hormones and steroids on brain functions, memory and learning, a similar role has been established for CRH (Croiset et al. 2000). In the process of establishing these functions, the presence of CRF receptors in the central nervous system and other tissues has been a starting point for investigations (reviewed by De Souza 1995; Perrin and Vale 1999; Eckard et al. 2002). As in other areas, specific animal models are now focusing on mutant mice for the prediction and confirmation of function (Contarino et al. 1999). Bruijnzeel and Gold (2005) have reviewed the role of CRF-like peptides in various forms of drug dependence, both to elucidate mechanisms and to explore potential therapies (Chatzaki et al. 2006). In keeping with the functional effect on the pituitary–adrenal system, involvement of CRH in the stress response has been addressed by Smith and Vale (2006), and the synthesis of CRF receptor antagonists may offer therapeutic potential in central disorders related to anxiety, psychiatric disorders, stress, and drug dependence (Valdez 2006).

Effects on the gastrointestinal tract of CRH have been described by several groups (Martinez and Tache 2006, 2007; Tache and Bonaz 2007).

Vergoni and Bertolini (2000) and Doyon et al. (2004) have suggested a functional role in the central regulation of appetite and body weight, by interaction with the melanocortin system.

Based on its interaction with CRH receptors, indications for modulation of synaptic transmission have

also been found with CRH (Orozco-Cabal et al. 2006), which has parallels with the newly discovered functional role of LH-RH (GnRH). Cardiovascular effects have been described and assigned to CRH receptors (Nazarloo et al. 2006). The existence of a CRH-like placental corticotropin-releasing factor has been confirmed even though the functional role remains elusive (Fadalti et al. 2000).

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N.9.4.2**In Vitro Assay for CRH Activity****PURPOSE AND RATIONALE**

The first *in vitro* assay procedures to detect CRF activity (Saffran and Schally 1955; Saffran et al. 1955) were based on incubation of rat anterior pituitary halves with the test substances in bicarbonate-buffered, oxygenated medium at 38°C for 1 h in the presence of 0.0004 M DL-arterenol and 0.004 M ascorbate followed by measurement of ACTH release into the medium by incubation with adrenal tissue and determination of corticosterone (a two-stage bioassay where a biological product of CRH action is determined by a quantitative biological response). Guillemin and Rosenberg (1955) used pituitary tissue culture from rat and dog and determined ACTH activity in the medium by the *in vivo* ascorbic acid depletion method in the rat according to Sayers (1954) (see Sect. N.7.4.1). The assay was later considerably simplified by using ACTH radioimmunoassay (RIA). Several modifications of these procedures have been used (Schally et al. 1968; Yasuda et al. 1982). Most investigators used rat pituitary cell cultures (Giguère and Labrie 1982; Giguère et al. 1982; Aguilera et al. 1983; Bilezikjan and Vale 1983; Vale et al. 1983a, 1983b; Patthy et al. 1985), while pituitary segments have been used by Antoni et al. (1983) and by Widmaier and Dallman (1984).

PROCEDURE

Anterior pituitaries are obtained from adult Sprague-Dawley rats, cut into small pieces and incubated in a Dubnoff incubator for 45 min at 37°C in 10 ml of oxygenated Medium 199 (GIBCO) containing 0.5% collagenase, 0.25% BSA, and 50 µg/ml gentamicin. The fragments can be easily dispersed mechanically into single cells by repeated suction and expulsion from a pipette. The cell suspension is centrifuged at room temperature for 10 min at 100 g. The cell pellet is then resuspended in 1.0 ml of medium and divided into four equal volumes. Each volume (containing about 5×10^6 cells) is mixed with 0.5 ml Sephadex G-15 which has been equilibrated previously with oxygenated medium. The mixture of pituitary cells and Sephadex is transferred into four chambers of a superfusion apparatus (Vigh and Schally 1984; Evans et al. 1988; Czernus and Schally 1991) consisting of a number of 1-ml plastic syringe barrels (modified by cutting off their distal end) mounted vertically in a Plexiglas holder which is kept at 37°C by circulating water. Each barrel is fitted with plungers at both ends.

Holes are drilled in the plungers to accommodate plastic tubing. The lower plunger is covered with a small piece of 30-µm-pore nylon net to keep the Sephadex beads from escaping. The “pores” between the beads are small enough to prevent the pituitary cells from escaping and large enough to allow unrestricted flow of medium through the column. The upper plunger tubing is used for directing the flow through the chamber from the medium reservoir. The flow through the system is controlled by a multichannel peristaltic pump which is placed after the superfusion chamber. Thus, the system is operated by suction with negative pressure.

The cells are perfused overnight with Medium 199 (Gibco) at a flow rate of 20 ml/h. The samples are administered every 30 min for 3 min and 1-ml fractions are collected at 3-min intervals. Synthetic CRF at 0.2 and 2 mM is used as standard. The corticotropin released into the medium is measured by a specific RIA for corticotropin.

EVALUATION

Dose-response curves are established for test preparation and standard allowing the calculation of potency ratios with confidence limits.

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N.9.4.3

In Vivo Bioassay of CRH Activity

PURPOSE AND RATIONALE

Munson and Briggs (1955) described an assay using rats pretreated with morphine and pentobarbital. Many modifications for a reliable *in vivo* bioassay of CRH activity have been tested including rats with hypothalamic lesions (Schally et al. 1968; Yasuda et al. 1982). Arimura et al. (1967) described an assay for corticotropin-releasing factor (CRF) using rats treated with morphine, chlorpromazine, dexamethasone and pentobarbital. This assay has been further improved by Graf et al. (1985).

PROCEDURE

Male Sprague-Dawley rats weighing about 200 g are maintained on a 12:12 h light:dark cycle at constant temperature for at least 5 days before use. On the day of the experiment, the rats are prepared with an initial single subcutaneous injection of a mixture of chlorpromazine (10 mg/kg) and morphine sulfate (20 mg/kg) followed after 75 min by pentobarbital (25 mg/kg) intraperitoneally. Then 60 min after the injection of pentobarbital, various doses of test substances or standard or vehicle (0.9% NaCl, 1% BSA, 0.1% ascorbic acid) are injected into an exposed jugular vein. Blood sam-

ples (0.5 ml) are collected in heparinized syringes immediately before and 30 min after injection of test substances. The blood is transferred to chilled polypropylene tubes containing EDTA (1.0 mg) and aprotinin (Trasylol, 50 μ l) and centrifuged (2000 g, 15 min 4°C). Plasma samples are stored at –20°C for corticosterone assay using a cross-reacting cortisol RIA that also measures corticosterone, the main corticoid secreted by rats, or a corticosterone RIA.

EVALUATION

Data for CRH activity of experimental groups are evaluated by analysis of variance or covariance followed by Duncan's multiple range test.

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N.9.4.4

Collection of Hypophyseal Portal Blood in Rats

PURPOSE AND RATIONALE

Worthington (1966), Porter and Smith (1967), Gibbs (1984), and Sarkar and Minami (1991) described methods to collect hypophyseal portal blood in rats. These methods can be used to measure the changes in secretion of several hypothalamic hormones under the influence of various factors.

Gibbs and Vale (1982), Gibbs (1985a), and Plotzky and Sawchenko (1987) studied the effect of various conditions on hypophyseal portal plasma levels of corticotropin-releasing factor (CRF) and arginine vasopressin.

Luteinizing hormone-releasing hormone (LHRH) was determined by Ching (1982), Sarkar (1987), Petraglia et al. (1987); thyrotropin releasing hormone (TRH) by Fink et al. (1982); growth-hormone-releas-

ing hormone (GH-RH) by Plotsky and Vale (1985); β -endorphin by Sarkar and Yen (1985); neuropeptide Y by Sutton et al. (1988); vasoactive intestinal peptide by Sarkar (1989); vasopressin and oxytocin by Gibbs (1984); and dopamine and epinephrine by Ben-Jonathan et al. (1977), and Gibbs (1985b).

PROCEDURE

Pentobarbital-anesthetized rats are placed on an isothermal heating pad in a stereotactic instrument. After cannulation of the trachea, the animals are mechanically ventilated. The base of the skull is exposed by a transpharyngeal approach, and the basisphenoid bone is drilled away. The dura is cut and deflected, the rat is heparinized, and, after the stalk is cut, a polyethylene cannula filled with saline is placed over the hypophyseal stalk for collection of blood. The cannula leads to an ice bath, and the distal end of the tubing ends in a fraction collector where portal blood is collected by gravity flow at a rate of about 10 μ l/min.

EVALUATION

Portal plasma concentrations of CRH, LHRH, TRH, GH-RH, β -endorphin, neuropeptide Y, vasoactive intestinal peptide, vasopressin and oxytocin, and catecholamines were determined after various treatments and compared with control periods of spontaneous secretion.

MODIFICATIONS OF THE METHOD

Cowell et al. (1991) described *in vitro* models for examination of the mechanisms controlling the secretion of hypothalamic hormones.

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N.9.4.5

CRH Receptor Determination

PURPOSE AND RATIONALE

CRH mediates its effects through high-affinity receptors identified and characterized by radioreceptor studies. Cellular signaling was measured by stimulation of adenylyl cyclase in membrane fractions of rat brain, pituitary, and spleen (Grigoriadis et al. 1993; Hauger and Aguilera 1993). Cloning studies indicate the existence of at least two types of mammalian receptor (De Souza et al. 1998; Steckler and Holsboer 1999; Dautzenberg and Hauger 2002). The CRF₁ receptor has been cloned from several species including human (Chen et al. 1993; Castro et al. 1996; Di Blasio et al. 1997; Grammatopoulos and Hillhouse 1998), mouse

(Vita et al. 1993), and rat (Perrin et al. 1993). Species homologies are 98% identical over the full length of 415 amino acids.

There are three isoforms of the CRF₂ receptor: the CRF_{2(a)} receptor, which has been cloned from both rat (Lovenberg et al. 1995) and human (Liaw et al. 1996), is a 411-amino-acid protein which shares approximately 70% identity with the CRF₁ receptor; the CRF_{2(b)} receptor isoform has been cloned from rat (Lovenberg et al. 1995), mouse (Stenzel et al. 1995), and human (Kostich et al. 1996) and is 431 amino acids in length, differing from the CRF_{2(a)} receptor in that the first 34 amino acids in the N-terminus are replaced by 54 different amino acids; the CRF_{2(c)} receptor has been identified in human brain (Sperle et al. 1997; Kostich et al. 1998).

[¹²⁵I]Sauvagine binding to CRH₂ receptors was characterized by Rominger et al. (1998).

CRH in plasma is mostly bound to the corticotropin-releasing hormone-binding protein (CRH-BP) and is therefore inactive except for the free fraction. CRH-BP is predominantly produced by the liver and distributed and expressed differently from the CRH receptors (Cortright et al. 1995; Zhao et al. 1997).

Rhode et al. (1996) used whole brains of Wistar rats for the CRH receptor assay.

PROCEDURE

Whole brains of male Wistar rats weighing 220–250g are homogenized with a Teflon-glass homogenizer (10 strokes at 800 rpm) in 0.32 M sucrose, 50 mM Tris/HCl (pH 7.2), 10 mM MgCl₂, 2 mM EGTA, and 0.15 mM bacitracin at 50 mg wet weight per milliliter. After centrifugation at 1000g for 5 min, the supernatant is centrifuged at 26,000g for 20 min. The pellet is resuspended in 50 mM Tris/HCl (pH 7.2), 10 mM MgCl₂, 2 mM EGTA, adding 0.15 mM bacitracin and 0.0015% aprotinin (assay buffer), and again centrifuged. The pellet is suspended in assay buffer containing 0.32 M sucrose and stored at –20°C. All steps are carried out at 4°C. Protein concentrations are determined by the method of Bradford (1976) using BSA as standard.

Then, 100 µg of membrane preparation in 300 µl assay buffer is incubated in quadruplicate with 0.1 nM [¹²⁵I]Tyr-oCRH in the absence and presence of 12 different concentrations (0.2 nM up to 1 µM) of unlabeled peptides at 25°C for 2 h. Nonspecific tracer binding is determined in the presence of 1 µM ovine CRH (oCRH). At the end of incubation, 3 ml of ice-cold assay buffer without inhibitors (containing

0.01% Triton X-100) is added to the assay tube and the samples are immediately filtered through GF/C filter discs (Whatman), presoaked for 2 h in 0.1% polyethylenimine using a Brandel-Harvester. The incubation tubes and filters are then washed with 3 ml cold washing buffer. Triton X-100 in this buffer strongly reduces nonspecific tracer binding. Radioactivity retained on the filter is measured by gamma-counting.

EVALUATION

Receptor affinities (K_{ass} , $K_d = 1/K_{\text{ass}}$) and capacities (B_{max}) are estimated using the nonlinear least-squares curve fitting program RADLIG (BIOSOFT, Cambridge, UK) and a K_d of 0.48 nM for the binding of the tracer peptide as determined from tracer saturation curves.

MODIFICATIONS OF THE METHOD

Schulz et al. (1996) used P2 membranes from human neuroblastoma IMR32 cells to test receptor binding of a selective nonpeptide antagonist of corticotropin-releasing factor (CRF).

Webster et al. (1996) used rat frontal cortex, pituitary, cerebellum, and heart tissue for *in vitro* characterization of antalarmin, a nonpeptide CRH receptor antagonist.

Grigoriadis et al. (1996) recommended ¹²⁵I-tyrosauvagine as a high-affinity radioligand for the pharmacological and biochemical study of human CRF 2 α receptors.

Differences between normal and adenomatous pituitary corticotrophs in the labeling characteristics of CRH receptors were reported by Abs et al. (1997).

Rabadan-Diehl et al. (1997) studied the role of glucocorticoids and hypothalamic factors in the regulation of pituitary CRH receptor mRNA and CRH during adrenalectomy in rats.

The differential regulation of hypothalamic pituitary CRH receptors during the development of adjuvant-induced arthritis in rats was reported by Aguilera et al. (1997).

Grammatopoulos and Hillhouse (1998) reported the solubilization and biochemical characterization of the human myometrial CRH receptor.

The interaction between glucocorticoids and CRH in the regulation of the pituitary CRH receptor has been studied in rats by Ochedalski et al. (1998).

The multiple actions of CRF on neuroendocrine and behavioral functions were examined using high-affinity, nonpeptide antagonists (Lundkvist et al. 1996).

Wei et al. (1998) described analogs of CRH and urocortin with selective activity at CRH₁ and CRH_{2β} receptors.

Perrin et al. (1999) used an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of CRF receptors.

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N.9.4.6

CRF Receptor Antagonists

PURPOSE AND RATIONALE

Studies with CRF antagonists indicate a role for CRF in certain psychiatric diseases and drug addiction (Koob 1999). This is in line with the known clinical psychotropic effects of corticotropin and glucocorticoids at elevated doses.

Various truncations of agonist peptides with deletions of the first 8–11 amino-terminal amino acid residues have resulted in potent peptide antagonists (Rivier et al. 1984; Hernandez et al. 1993; Miranda et al. 1994; Schulz et al. 1996). Several nonpeptide CRF receptor antagonists have been described (Griebel et al. 1998; Okuyama et al. 1999; Habib et al. 2000; Gully et al. 2002; Griebel et al. 2002; Heinrichs et al. 2002; Grigoriadis 2003; Gutman et al. 2003). Receptor autoradiography was performed in treated rats by Heinrichs et al. (2002) and Gutman et al. (2003).

PROCEDURE

Brains from treated rats are sectioned at the level of the prefrontal cortex and lateral septum to determine CRF₁ and CRF₂ receptor binding, respectively. The prefrontal cortex has been shown via *in situ* hybridization to express predominantly CRF₁ receptors, whereas the lateral septum expresses CRF₂ receptors in rodents (Van Pett et al. 2000). After the behavior experiments, 15- μ m-thick rat brain sections containing the prefrontal cortex and the lateral septum are sectioned at approximately -20°C , mounted on Superfrost Plus Slides and stored at -80°C until the

assay. *Ex vivo* CRF receptor autoradiography is performed on 15- μ m-thick rat brain sections. Sections are removed from the -80°C freezer and allowed to warm up to room temperature in a desiccator. Brain sections are fixed for 2 min in 0.1% paraformaldehyde, pH 7.5, followed by a 15-min incubation in assay buffer (50 mM Tris, 10 mM MgCl₂, 2 mM EGTA, 0.1% bovine serum albumin, 0.1 mM bacitracin, and 0.1% aprotinin, pH 7.5). Triplicate slides containing adjacent brain sections are incubated in one of three conditions:

1. 0.1 nM radiolabeled ¹²⁵I-sauvagine to determine total binding at both the CRF₁ and CRF₂ receptor subtypes;
2. 0.1 nM radiolabeled ¹²⁵I-sauvagine + 1 μ M of a specific CRF₁ receptor antagonist to be tested, to determine CRF₂ receptor-specific binding; or
3. 0.1 nM radiolabeled ¹²⁵I-sauvagine + 1 μ M unlabeled sauvagine to determine nonspecific binding.

After a 2-h incubation, unbound radioligand is removed by two 5-min rinses in ice-cold phosphate-buffered saline + 1% bovine serum albumin on a rotating platform at 60 rpm, followed by two brief dips in ice-cold distilled, deionized H₂O. Slides are then rapidly dried using a cold air blow dryer and exposed to Kodak Biomax MR film with ¹²⁵I-microscale standards (Amersham Biosciences) for 80–90 h.

Images from the receptor autoradiography films are digitized with a CCD-72 (Dage-MTI, Michigan City, Ind., USA) image analysis system equipped with a Nikon camera. Semiquantitative analysis is performed using AIS software (version 4.0, Imaging Research, St. Catharines, ON, Canada). Optical densities are calibrated against co-exposed ¹²⁵I-microscale standards and expressed in terms of nanocuries per gram of tissue equivalent.

EVALUATION

CRF₁-receptor-specific binding is calculated as total binding minus CRF₂ receptor binding, and CRF₂ receptor-specific binding is calculated as CRF₂ receptor binding minus nonspecific binding. Receptor binding data are expressed as a percentage of the control values. The percentage of binding is subtracted from 100 to estimate percentage of occupancy by the antagonist relative to vehicle-treated rats. In all cases, three to four sections are matched at the rostrocaudal level and used to produce a single value for each animal. Significant differences are evaluated by one- or two-tailed *t* tests or one-way ANOVA followed by Student-Newman-Keuls post hoc analysis.

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N.9.5**Growth-Hormone-Releasing Hormone (GH-RH)****N.9.5.1****General Considerations**

The discovery of the growth-hormone-releasing hormone (GH-RH) by physiological experiments, and the isolation and purification of the hormonal principle are reviewed elsewhere (Reichlin et al. 1976; Krulich and Fawcett 1977; Ling et al. 1985; Frohman and Jansson 1986; Gelato and Merriam 1986; Grossman et al. 1986; Thorner et al. 1986; Frohman et al. 1986; Muller 1987; Vance 1990). The initial studies on analogs of GH-RH raised expectations that therapy by GH injections might be replaced by GH-RH therapy (Dean and Friesen 1986). It was however found that infusions and high-dose treatment with GH-RH reduced to the response of GH release, in a similar manner as described for the gonadotropin receptors, due to receptor adaptation (Catt et al. 1980).

The comparative endocrinology of hypothalamic control of GH secretion by GH-RH, and the presence of GH-RH receptors in animal species have been explored for mammalian and nonmammalian animal species (Ukena et al. 2006; Lee et al. 2007).

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N.9.5.2

Radioreceptor Assay of Growth Hormone-Releasing Hormone

PURPOSE AND RATIONALE

As for other polypeptide hormones, GH-RH receptors may be used for quantification and potency estimates of relative binding affinities. Several agonistic and antagonistic analogs of human GH-RH (hGH-RH) have been synthesized by various groups of investigators. The clinical and basic aspects of GH-releasing peptides are reviewed by Argente et al. (1996).

In vitro assays with pituitary cells have been used for screening the biological activity of hGH-RH analogs prior to their evaluation *in vivo* (Heiman et al. 1985; Kovacs et al. 1988; Campbell et al. 1991). A careful determination of binding activities of the peptides to specific GH-RH receptors provides important data for the design of more active analogs. Several studies demonstrated that the radioligand, [His¹-, ¹²⁵I-Tyr¹⁰, Nle²⁷]-hGH-RH(1-32)NH₂, binds specifically to a single class of receptors in rat pituitary cells and homogenates, and can be used for radioreceptor assay (Campbell et al. 1991; Seifert et al. 1985a, 1985b; Struthers et al. 1989). The use of a radioreceptor assay for *in vitro* screening of analogs of GH-RH was described by Halmos et al. (1993).

PROCEDURE

Pituitaries from male Sprague-Dawley rats (250–300 g) are used to prepare crude membranes. Immediately after decapitation, anterior pituitaries are removed, rinsed with cold saline, and homogenized in 5 times their volume of homogenization buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM MgCl₂, 30 μg/ml bacitracin, pH 7.4) on ice using an Ultra-Turrax homogenizer at maximal speed. The homogenate is centrifuged at 500 g for 10 min at 4°C. The supernatant containing the crude membrane fraction is again centrifuged at 70,000 g for 50 min at 4°C. The pellet is washed twice by resuspending in ice-cold homogenization buffer and spinning. The final pellet is resuspended in homogenization buffer and stored at –70°C until used for the receptor binding studies. Protein concentration is determined by the method of Bradford (1976) using a commercially available protein assay kit.

[His¹, ¹²⁵I-Tyr¹⁰, Nle²⁷]hGH-RH(1-32)NH₂ is iodinated by the chloramine-T method (Greenwood et al. 1963). The receptor binding assay is carried out for 60 min in GH-RH binding buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM MgCl₂, 1% BSA, 30 μg/ml bacitracin, pH 7.4) at 24°C. For saturation binding analyses, 50–150 μg membrane homogenates are incubated in duplicate with a least six concentrations of radioligand, ranging from 0.005 to 0.35 nM in the presence or absence of excess unlabeled peptide (1 μM) in a final volume of 300 μl. Incubation is terminated by immersing siliconized borosilicate tubes in ice water, transferring 270 μl of the suspension into cold siliconized polypropylene microfuge tubes, and centrifuging at 12,000 g for 2 min at 4°C, and aspirating the supernatants. To reduce the nonspecific binding, 500 μl/tube of GH-RH binding buffer is added and the tubes re-centrifuged. The washing step is repeated and the supernatant again aspirated. Finally, the bottoms of the tubes, containing the pellet, are cut off and counted for radioactivity in a gamma-counter.

In the competition experiments, which also include the specificity experiments, 50–100 μg of membrane homogenates are incubated in duplicate with 0.10–0.15 nM radioligand plus various concentrations of nonradioactive analogs and other peptides (10⁻¹²–10⁻⁶ M).

EVALUATION

Percent specific binding is plotted against the log concentration of competitors. The curves are compared at the 50% specific binding levels (IC₅₀). The ligand-PC and McPherson computerized curve fitting pro-

grams of Munson and Rodbard (1980) are used to analyze competition and saturation data. To determine the types of receptor binding, dissociation constants (K_d) and the maximal binding capacity of receptors (B_{max}), and the saturation binding data are also analyzed by the Scatchard method.

MODIFICATIONS OF THE METHOD

Carrick et al. (1995) described a rapid and sensitive binding assay for GH releasing factor (GRF). Human embryonic kidney (HEK293) cells and rat pituitary tumor (GH₄C₁) cells were transfected with the porcine GRF receptor cDNA. Stably expressing cell lines are referred to as 293-P2 and GH₄-P1, respectively. GH₄C₁ cells transfected with somatostatin receptor subtype 2 are called GH₄-R2.20 and HEK293 cells transfected with somatostatin receptor subtype 5 are called 293-R5.2. GH₄-P1 and 293-R5.2 were derived by the calcium phosphate method of transfection and 293-P2 and GH₄-R2.20 using lipofectamine. For binding assays, cells were removed from culture dishes by the addition of phosphate-buffered saline containing 1 mM EDTA and cell membrane fractions prepared. Membrane pellets were suspended in 25 mM Tris, pH 7.4, plus protease inhibitors, aliquoted and stored at -80°C . The binding assay consisted of approximately 25 pM radiolabeled ligand in the presence or absence of unlabeled ligand in assay buffer (50 mM HEPES, pH 7.4, 5 mM MgCl₂, 2 mM EGTA) and cell membranes (2–10 μg). The total assay volume of 200 μl included wheat-germ-agglutinin-coated SPA beads at a concentration of 1 mg/assay, and for GRF binding, 0.05 mg/ml alamethicin (Sigma St. Louis, Mo., USA); 96-well plates were used for most assays. Assays were agitated on a plate shaker for 3 h. Samples were counted on a Wallac Microbeta Counter for 1 min per sample. The conventional binding assay was performed (Mayo 1992), except that following binding and centrifugation the membranes were washed in 0.5 ml binding buffer, centrifuged, and counted.

Abribat et al. (1990) and Gaudreau et al. (1992) developed a binding assay for GH-RH in rat pituitaries. Using this technique, Lefrancois and Gaudreau (1994) tried to identify the receptor-binding pharmacophores of GH-RH in rat pituitaries.

Hassan et al. (1995) developed a competitive binding assay using cloned porcine GH-RH receptors in order to study structure–activity relationships.

Kajikowski et al. (1997) investigated GH-RH receptor structure and activity using yeast expression technologies.

Muccioli et al. (1998) characterized specific binding sites for synthetic GH secretagogues (sGHS) on membranes from pituitary gland and different human brain regions using a peptidyl sGHS (Tyr-Ala-hexarelin) which has been radio-iodinated to high specific activity at the Tyr residue.

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N.9.5.2.1

Growth Hormone Release from Rat Pituitaries *in Vitro*

PURPOSE AND RATIONALE

For testing of pituitary cell stimulation, isolated pituitary glands or cell cultures may be used. Antagonism by adding somatostatin, for example, is tested in the presence of a standard concentration of GH-RH. Human GH-RH has the structure of a 44-amino-acid amide, which was isolated from human endocrine tumors and later confirmed as a hypothalamic hormone. The effect of GH-RH analogs can be tested on isolated rat pituitaries measuring GH release. This test system avoids the interference of counter-regulatory somatostatin secretion, which limits the duration of GH release *in vivo*.

PROCEDURE

The pituitaries of male Sprague-Dawley rats weighing about 100 g are quickly removed after decapitation. The posterior lobe is discarded and the anterior lobe is divided into two halves by a midsagittal cut. Five bisected hemipituitaries are incubated in plastic vials containing 4 ml TCM 199 with 0.1% BSA, 15 µg/ml penicillin, and 25 µg/ml streptomycin. The vials are gassed with 95% O₂ and 5% CO₂. After 30 min of control incubation, the medium is changed and various doses of standard and test substances are added for an incubation of 90 min. GH content in the medium and in the pituitaries is determined by a specific radioimmunoassay (Schalch and Reichlin 1966).

EVALUATION

Dose–response curves are established for standard and test compounds measuring GH release into the medium and GH depletion from the pituitaries allowing calculation of potencies ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Superfused pituitary cells may be used measuring activity and duration of effect, as well as interaction of

stimulatory and inhibitory factors. Growth-hormone-releasing factor from tumors in human pancreas and from rat hypothalami as well as analogs of GH-RH were evaluated in a superfused pituitary cell system (Vigh and Schally 1984; Czernus and Schally 1991; Halmos et al. 1993), see also Sect. N.9.4.2, *in vitro* assay for CRH activity. Anterior pituitaries of two young adult male Sprague-Dawley rats were digested with 0.5% collagenase CLS2 (Worthington) for 50 min. After incubation, the fragments were digested into cell clusters (5–40 cells) by mechanical dispersion, and then transferred onto two columns and allowed to sediment simultaneously with 0.8 ml Sephadex G-10. The dead volume of the system was set to 1 ml. Medium 199 containing BSA (2.5 g/l), NaHCO₃ (2.2 g/l) and gentamicin sulfate (85 µg/ml) was equilibrated with a mixture of 95% air and 5% CO₂ and used as the culture medium. The medium was pumped at a flow rate of 0.33 ml/min. During an overnight recovery period, the baseline stabilized and the cells regained their full responsiveness. The samples were then infused through a four-way valve at 5×10^{-10} M concentration for 3 min (1 fraction) at 45-min intervals. Rat GH was determined by double-antibody radioimmunoassay.

The same system was used by Rekasi and Schally (1993) and Kovács et al. (1996a) to evaluate the activity of GH-RH antagonists. For determination of the antagonistic activity, the cells were exposed to 10^{-8} , 10^{-7} , and 10^{-6} M GH-RH antagonist simultaneously with 10^{-9} M GH-RH or to 10^{-6} M GH-RH antagonist combined with 100 mM KCl (controls for potassium-stimulated GH secretion) for 3 min. After 30 min, the duration of the inhibitory effect of GH-RH antagonist was also tested by repeated 3-min infusions of 10^{-9} M GH-RH.

Using this system with pituitaries of **transgenic mice** overexpressing the human GH-RH gene, Kovacs et al. (1997) evaluated the effects of GH-RH antagonists.

In addition to growth hormone release, Horváth et al. (1995) determined cAMP release from superfused rat pituitary cells stimulated by GH-RH.

GH release was determined using cultured rat pituitary cells (Brazeau et al. 1982; Perkins et al. 1983; Scheikl-Lenz et al. 1985). Pituitary cells were prepared by enzyme dispersion with collagenase, DNase and pancreatin. The cells were cultured for 3 days in microbiological Petri dishes in Dulbecco's modified essential medium with 20 mM HEPES, 15% fetal calf serum, 100 mU/ml penicillin-G and 100 µg/ml streptomycin at 37°C and 10% CO₂.

Cheng et al. (1993) tested time- and dose-dependent GH release by a nonpeptidyl GH secretagogue in rat pituitary cells.

Sanchez-Hormigo et al. (1998) tested GH-releasing hexapeptide, one of several synthetic GH secretagogues, on GH secretion from cultured porcine somatotropes.

Kovács et al. (1996b) measured the effects of chronic administration of a GH-RH agonist on body weight, tibia length and tail length in growth-hormone-deficient (monosodium-glutamate-lesioned) rats.

Jacks et al. (1996) evaluated an orally active GH secretagogue in dogs. Serum GH levels were dose-dependently increased after oral and intravenous administration. Moreover, an increase of insulin-like growth factor and serum cortisol was found.

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N.9.5.2.2

Long-Term Pituitary Cell Culture

Long-term-cultured pituitary cells have been used extensively to study the response of GH secretion and synthesis. Roh et al. (2006) applied this principle to the investigation of priming by GH-RH and by synthetic secretagogues derived from ghrelin. They found that low doses of GHRP-2 and GH-RH were suitable for priming somatotrope cells in culture for subsequent stimulation by GH-RH and ghrelin.

Studies *in vitro* using long-term cell cultures have shown that priming with low doses of GH-RH enhances the subsequent response to the stimulation with GH-RH analogs. Fintini et al. (2005) have used a new animal model for the assessment of GH secretion, the GH-deficient GH-RH-knockout (GH-RH-KO) mouse model. They found that priming with the non-GH-RH secretagogue derived from the gastrointestinal peptide ghrelin augmented the subsequent response to a GH-RH analog.

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N.9.5.3

GH-RH Bioassay by Growth Hormone Release in Rats

PURPOSE AND RATIONALE

Releasing activity can be determined by plasma GH stimulation after intravenous or subcutaneous injection of GH-RH in rats.

PROCEDURE

Male Sprague-Dawley rats weighing about 100 g are anesthetized by fractionated subcutaneous injections of 0.7 ml/kg of 25% urethane solution. The jugular vein is cannulated. Various doses of GH-RH test preparations or standard are injected intravenously in 0.2 ml 1% gelatin/saline. After 15 min blood samples are collected for determination of GH by a specific radioimmunoassay (Schalch and Reichlin 1966). The plasma concentration is expressed in terms of NIAMD-rat-GH-RP-1.

As a modification also suitable for testing other releasing hormones, e. g., by subcutaneous application, a double-barreled polyethylene catheter is implanted into the jugular vein, with one lumen being used for blood sampling and the second small lumen being used to keep the catheter open by heparin infusion into the tip of the collection catheter. Blood is continuously withdrawn by a peristaltic pump and fractions of 500 μ l heparinized blood are collected at intervals of 10 min. To compensate for blood losses, the erythrocyte fraction of four to eight consecutive plasma samples is re-infused in a plasma expander (Haemaccel 3.5%) via a femoral vein. Before treatment, two 2-min samples are collected, and, after injection, two 2-min samples are collected again, followed by sampling intervals of 10 min for up to 3 h. GH is determined by a specific radioimmunoassay (Schalch and Reichlin 1966).

EVALUATION

Using several doses of standard and test preparation dose–response curves are established, allowing calculation of the potency ratio with confidence limits. Time–concentration curves after subcutaneous or, for

example, intranasal application allow the duration of action to be evaluated.

MODIFICATIONS OF THE METHOD

Wehrenberg and Ling (1983) and Wehrenberg et al. (1985) determined the *in vivo* biological potency of rat and human GH-releasing factor and fragments of human GH-releasing factor in swivel-cannulated, conscious, freely moving rats and in cannulated anesthetized rats.

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N.9.5.4

GH-RH Analogs

Many studies have been performed with synthetic GH-RH analogs, starting with the active sequence GHH (1–29), and proceeding to more powerful compounds, which were explored in domestic animals (Bhatti et al. 2006), and tested for their therapeutic potential in clinical studies (Doi et al. 2004; Furuta et al. 2004; Veldhuis et al. 2005).

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N.10**Other Peptide Hormones****N.10.1****Melatonin****N.10.1.1****General Considerations**

The role of melatonin as a neurotransmitter, a hormone, and a possible therapeutic agent has been a matter of controversy (Brainard 1978; Reiter 1991; Hajak et al. 1996; Huether 1996; Jansen et al. 2006) ever since melatonin was isolated and identified as *N*-acetyl-5-methoxytryptamine by Lerner et al. (1958) and characterized by Axelrod and Wurtman (1966). Melatonin is synthesized and released by the pineal gland and has been shown to play a key role in the regulation of mammalian circadian rhythms and reproductive functions (Reiter 1991; Arendt et al. 1995; DiBella and Gualano 2006). Melatonin is also produced in extrapineal sites, such as the retina (Lundmark et al. 2006), the Harderian glands, and the gut (Huether 1993, 1994; Messner et al. 2001). It should however be noted that methods for the identification of peptides and neurotransmitters have reached extremely high levels of sensitivity; as a consequence, their presence in a tissue does not necessarily indicate that a relevant physiological function is subserved in that organ or tissue. Melatonin is synthesized from 5-hydroxytryptamine (5-HT) by a two-step biochemical pathway (Reiter 1991). Initially, 5-HT is acetylated to produce *N*-acetylserotonin, which is subsequently *O*-methylated to form melatonin.

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N.10.1.2**Melatonin Receptor Binding****PURPOSE AND RATIONALE**

The activity of melatonin is mediated through high-affinity G-protein-coupled receptors. Two human melatonin receptor subtypes, hMT₁ and hMT₂, have been cloned (Reppert et al. 1994, 1995; Browning et al. 2000; Dubocovich et al. 2001). A third melatonin-binding site named MT₃ has been described as the human homolog of cytoplasmatic quinone reductase 2 (Nosjean et al. 2000, 2001).

Audinot et al. (2003) described the evaluation of selective ligands of human cloned MT₁ and MT₂ receptors. Binding affinities were determined using [²⁻¹²⁵I]-melatonin. Methods for the measurement of melatonin and melatonin derivatives (Middleton 2006) and for the melatonin receptor ligands (Zlotos 2005) have been reviewed

PROCEDURE**Membrane Preparations**

HEK.293 and CHO-K1 cell lines stably expressing the hMT₁ or hMT₂ receptor are grown to confluence, harvested in phosphate buffer containing 2 mM EDTA and centrifuged at 1000 g for 5 min (4°C). The resulting pellet is suspended in 5 ml Tris/HCl, pH 7.4, containing 2 mM EDTA and homogenized using a Kinematica polytron. The homogenate is then centrifuged (20,000 g, 30 min, 4°C), and the resulting pellet is suspended in 75 mM Tris/HCl, pH 7.4, containing 2 mM EDTA and 12.5 mM MgCl₂. Aliquots of membrane preparations are stored in binding buffer (Tris/HCl 50 mM, pH 7.4, 5 mM MgCl₂) at –80°C until use.

[²⁻¹²⁵I]Melatonin Binding Assay

Membranes are incubated for 2 h at 37°C in binding buffer (Tris/HCl 50 mM, pH 7.4, 5 mM MgCl₂) in

a final volume of 250 μ l containing [2-¹²⁵I]melatonin (200 pM) for competition experiments using CHO cells and 25 or 200 pM, respectively, for MT₁ and MT₂ receptors expressed in HEK cells. Nonspecific binding is defined with 10 μ M melatonin. The reaction is stopped by rapid filtration through GF/B unifilters, followed by three successive washes with ice-cold buffer.

EVALUATION

Data are analyzed using the program PRISM (GraphPad Software, San Diego, Calif., USA). For saturation assays, the density of binding sites (B_{\max}) and the dissociation constant of the radioligand (K_D) are calculated according to the method of Scatchard. For competition experiments, inhibition constants are calculated according to the Cheng-Prusoff equation. For the correlation analysis of pK_i values, the Pearson product moment correlation coefficient is employed.

MODIFICATIONS OF THE METHOD

Receptor binding assays have been used to evaluate new melatonin receptor ligands (Depreux et al. 1994; Dubocovich et al. 1997a; Charton et al. 2000a, 2000b; Nonno et al. 2000).

Dubocovich et al. (1997b) used **rabbit retina membranes** for binding studies with [2-¹²⁵I]melatonin.

Nonno et al. (2000) and Audinot et al. (2003) used the [³⁵S]GTP γ S binding assay to evaluate the functional activity of agonists and antagonists. Membranes were prepared as for the [2-¹²⁵I]melatonin binding assay. Together with test drugs, they were diluted in binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 3 mM MgCl₂, 3 μ M GDP) in the presence of 20 μ g/ml saponin in order to enhance the agonist-induced stimulation level. For agonist tests, incubation was started by the addition of 0.1 nM [³⁵S]GTP γ S to membranes and ligands, and carried on for 60 min at room temperature in a final volume of 250 μ l. To test for antagonistic activity, membranes were preincubated for 30 min with melatonin (30 nM or 3 nM for hMT₁ and hMT₂ receptors, respectively) and several concentrations of the tested compound. The reaction was started by the addition of 0.1 nM [³⁵S]GTP γ S and followed by a 60-min incubation. Nonspecific binding was assessed using nonradiolabeled GTP γ S (10 μ M). All reactions were stopped by rapid filtration through GF/B unifilters presoaked with distilled water, followed by three successive washes with ice-cold buffer.

Data were analyzed using the program PRISM to yield EC₅₀ and E_{\max} values for agonists. Antagonist potencies were expressed as K_B with $K_B =$

IC₅₀/1+([ago]/EC₅₀ago), where IC₅₀ is the inhibitory concentration of antagonist that gives 50% inhibition of [³⁵S]GTP γ S binding in the presence of a fixed concentration of agonist ([ago]) and the EC₅₀ago is the EC₅₀ of the agonist when tested alone. I_{\max} (maximal inhibitory effect) was expressed as a percentage of that observed with melatonin, 30 nM and 3 nM ([ago]) for hMT₁ and hMT₂ receptors, respectively.

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N.10.1.3

In Vitro Assay of Melatonin: Inhibition of Forskolin-Stimulated cAMP Accumulation

PURPOSE AND RATIONALE

Reppert et al. (1995), Browning et al. (2000), and Charton et al. (2000a, 2000b) used the inhibition of forskolin-stimulated cAMP accumulation for characterization of melatonin receptors and evaluation of synthetic derivatives.

PROCEDURE

Generation of CHO-mt₁ and CHO-MT₂ Cells

Clones representing the sequence of the human mt₁ receptor are amplified using degenerate primers based on the sequence of the *Xenopus laevis* melatonin receptor (Ebisawa et al. 1994). Cloning was in pBlue-script (Stratagene, La Jolla, Calif., USA). The human MT₂ sequence is cloned in a similar manner using the sequence described by Reppert et al. (1995). The sequences encoding the receptor are cloned into the mammalian vector pcDNA3 (Invitrogen) and introduced into CHO cells by conventional calcium phosphate precipitation techniques, which are then placed under G418 selection (1 mg/ml). Cell lines, which give the greatest melatonin-induced inhibition of cAMP accumulation, are chosen for further studies.

cAMP Assays

The assay is performed using 96-well plates in a final assay volume of 200 µl. Confluent CHO-mt₁ and CHO-MT₂ cells are incubated at 37°C with DMEM-F12, containing 300 µM isobutylmethylxanthine to inhibit phosphodiesterase activity. Following a 60-min incubation, agonist (0.01 pM to 100 µM) is added. After 60 min, forskolin (30 µM) is added and cells are incubated for a further 15 min. For antagonist experiments, melatonin is co-incubated with luzindole (0.1–100 µM) for 60 min prior to the addition of forskolin. The reaction is terminated by removal of media and addition of ice-cold ethanol (100 µl) for 30 min at 4°C. Ethanol samples are evaporated and

cAMP concentrations determined by [¹²⁵I]cAMP scintillation proximity assay (Amersham).

EVALUATION

cAMP data are fitted with a four-parameter logistic equation to determine pIC₅₀ values and Hill coefficients. Drug responses are expressed as percentage inhibition of forskolin-stimulated cAMP. The potency ratio is defined as the ratio of the EC₅₀ of the drug relative to that of melatonin determined in the same experiment.

MODIFICATIONS OF THE METHOD

Morgan et al. (1989) and Conway et al. (2000) studied inhibition of cAMP production in cultures of ovine pars tuberalis cells by melatonin.

Furthermore, Conway et al. (2000) characterized the human melatonin mt₁ and MT₂ receptors using the **CRE-luciferase reporter assay**. HEK293 cells expressing human melatonin mt₁ and MT₂ receptors were cultured to ~80% confluence at which time they were co-transfected with the plasmids pGL3 (CRE2-TK) and pcDOR8 (Barrett et al. 1994) using the lipid transfection reagent FuGENE™ 6 (Boehringer Mannheim). Cells were cultured for 24 h post-transfection before being washed with phosphate-buffered saline (pH 7.4), recovered in phenol-red-free Dulbecco's modified Eagle medium (supplemented with 10% fetal calf serum) and seeded as 50-µl aliquots (5 × 10⁴ cells) into white 96-well tissue culture plates. Following a further 24-h incubation, drug treatments were added in a 5.6 µl volume to triplet wells, and plates were left to incubate overnight (16 h). The level of expressed luciferase was measured by the addition of 50 µl of Constant Light Signal Reagent (Boehringer Mannheim) to each well, and plates were briefly placed in the dark (30 min, room temperature) before being quantified on a Packard LumiCount™ Reader (1 s per well). Where experiments were designed to measure drug-mediated dose–response effects, a control melatonin dose–response study was always included. Data from the triplet wells were averaged and the control data normalized from a value of 100 (no melatonin) to a value of 0 (maximum response, 10⁻⁵ M melatonin). Data obtained from other drug treatments were normalized relative to the control values determined in that experiment. All experiments were performed on three or more separate occasions, and mean values ± SEM determined.

IC₅₀ values and apparent efficacies were calculated by fitting a four-parameter logistic curve through the

mean normalized data. For antagonists, pA_2 values were determined by performing Schild analysis.

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N.10.1.4

Dopamine Release from Rabbit Retina

PURPOSE AND RATIONALE

Dubocovich et al. (1997) compared the pharmacological profile of the rabbit retina ML_1 presynaptic heteroreceptor that mediates the calcium-dependent release of dopamine to that of the human Mel_{1a} and Mel_{1b} recombinant melatonin receptors, and determined potencies of melatonin receptor agonists and antagonists.

PROCEDURE

Albino rabbits (2.5–3.5 kg) maintained on a 14-h/10-h light/dark cycle are sacrificed by decapitation during the light cycle. Retinal pieces are incubated in Krebs solution gassed with 95% O_2 and 5% CO_2 for 20 min at 37°C in the presence of 0.1 μM [3H]dopamine (specific activity 55 Ci/mol). The pieces of retina are washed in

Krebs solution at 37°C and placed in glass superfusion chambers between a pair of platinum electrodes 30 mm apart. Retinal tissue is superfused at 1 ml/min with Krebs solution (37°C) containing (*S-R*)-sulpiride (0.1 μM) to block D_2 dopamine autoreceptors, beginning 60 min after the onset of superfusion, i. e., when the spontaneous outflow of radioactivity has leveled off. Samples of the superfusate are collected by means of a fraction collector at 4-min intervals. Tritium release from retinal tissue is elicited by field stimulation using 2-min-duration (3 Hz, 20 mA, 2 ms) pulses that are delivered from platinum electrodes by a Grass stimulator. During stimulation, pulses are monitored on an oscilloscope. In each experiment two periods of field stimulation are applied at 60 min and 100 min after the incubation with [3H]dopamine. Samples of the superfusate are collected before, during, and after the period of stimulation. Drugs are added to the perfusion medium either 40 min before the first or 20 min before the second stimulation period. At the end of the experiment, the retinal tissue contained in each chamber is solubilized in 0.5 ml TS-1 (Research Products Int., Elk Grove, Ill., USA) and the tritium content is determined by liquid scintillation counting.

EVALUATION

The outflow of radioactivity in each sample is expressed as the percentage of total tissue radioactivity determined to be present at the beginning of each sample collection period: total tritium released per sample divided by total tritium present in the tissue all multiplied by 100 (Dubocovich 1985). Results are expressed as the ratio of the overflow during the second and first periods of field stimulation within the same experiments.

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N.10.1.5

Effect on *Xenopus* Melanophores

PURPOSE AND RATIONALE

TeckTeh and Sugden (1998) examined the potency of melatonin receptor ligands using the pigment aggregation response in a clonal line of *Xenopus laevis* melanophores.

PROCEDURE**Culture of *Xenopus laevis melanophores***

A clonal line of *Xenopus laevis melanophores* (M. Lerner, University of Texas) is grown in diluted (0.7×) L-15 medium (Sigma) containing 20% heat-inactivated fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The medium is first conditioned by *Xenopus laevis* fibroblasts (Daniolos et al. 1990).

Microtiter plate assays

Melanophores are seeded into flat-bottomed 96-well microtiter plates and grown in conditioned L-15 medium at 24°C for 2–4 days until cell density is (6–8)×10⁴ cells/well. For the 4–18 h before using melanophores, condition medium is replaced with 0.7× L-15 medium; pigmented granules remain fully dispersed in this medium. Agonist potency is determined by measuring the redistribution of pigment granules within the melanophores by quantitating the change in absorbance of the cells at 630 nm on a Biotek microtiter plate reader. The fractional change in absorbance (1 – [A_f/A_i], where A_i is the initial absorbance before drug addition and A_f is the final absorbance 1 h after drug treatment) is calculated for each concentration of drug tested.

EVALUATION

Concentration–response data are plotted and EC₅₀ values are determined using the ALLFIT program (De Lean et al. 1978) with the four-parameter logistic equation:

$$Y = [A - D]/[1 + (X/C)B] + D$$

where *X* is the concentration of the analog, *Y* is the fractional change in absorbance, *A* is the maximal absorbance in the absence of the analog, *B* is the slope factor, *C* is the concentration of the analog producing half of the maximal response (EC₅₀) and *D* is the minimal absorbance. The response to each drug is measured in six independent experiments. Antagonistic potency (pA₂) is determined by constructing concentration–response curves to melatonin in the absence or presence of varying concentrations (1–100 µM) of each antagonist.

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N.10.1.6**Vasoconstrictor Activity of Melatonin****PURPOSE AND RATIONALE**

Evans et al. (1992), Ting et al. (1997), and Delagrangé et al. (1999) used the vasoconstrictor action of melatonin in the tail artery of juvenile Wistar rats to evaluate melatonin agonists and antagonists. Two different recording systems, the Halpern pressure myograph (Halpern et al. 1984) and the Halpern–Mulvany wire myograph, were compared.

PROCEDURE

Male juvenile (3–4 weeks old, weighing 55–100 g) Wistar rats are housed in a 12-h light/dark cycle. They are sacrificed 1–2 h after lights on by decapitation. The ventral artery of the tail is dissected and placed onto a dissecting disc in gassed, modified Krebs–Henseleit solution. The blood vessel is carefully cleaned from fat and connective tissues with the aid of a dissecting microscope and divided into ring segments of 2–3 mm length. Ring segments are suspended between two supporting jaws in a stainless steel chamber of a Mulvany–Halpern wire myograph and allowed to equilibrate for 30 min. The preparations are bathed in Krebs–Henseleit solution maintained at 34 ± 1°C and gassed with 95% O₂/5% CO₂. Tension is applied by adjusting the micrometer connected to one of the supporting jaws, while an isometric transducer connected to the other jaw measures force. An initial tension of 0.2–0.5 g is applied. Then the preparations are allowed to relax to 0.1–0.4 g weight. Vessels are contracted with 60 mM KCl to assess tissue viability and provide a reference contracture for subsequent data analysis. A stimulator is used to deliver 5-s trains of electrical pulses (10–20 V, 3 ms pulse width) at a frequency of 2–3 Hz every 4–5 min. The voltage and the frequency of the field stimulation are adjusted at the beginning of each experiment to obtain 20%–35% of the KCl response. Concentration–responses curves are constructed for melatonin and test compounds. For experiments with putative antagonists, these agents are added 20 min before the construction of a dose–response curve for melatonin.

EVALUATION

Isometric tension responses are expressed as a percentage of the enhancement in response to the predrug of

neurogenic contractions. The sensitivity of the preparations is assessed as the negative logarithm of the concentration required to produce 50% of the maximum response (pEC_{50}) after the agonist concentration–effect ($E/[A]$) data are fitted to the formula:

$$E = \alpha[A]^n / ([A]^n + [A_{50}]^n),$$

where E is the response, α is the asymptote, $[A]$ is the agonist concentration, n is the gradient of the $E/[A]$ curve and $[A_{50}]$ is the midpoint of the $E/[A]$ curve (Black et al. 1985). $[A_{50}]$ values represent agonist concentration giving 50% of the maximum responses and are shown as the negative logarithm (pEC_{50}). The maximum response of each analog is expressed as a ratio of the maximum response to melatonin (E_{max}) obtained in segments from the same animal. For the antagonistic experiments, the agonist concentration ratio (CR) is determined in each experiment. The CR is the ratio of EC_{50} values in the presence and absence of antagonist. Differences between mean values are compared using either paired or unpaired Student's t -test (two tailed) and are considered statistically significant if $P < 0.05$.

MODIFICATIONS OF THE METHOD

Viswatanan et al. (1997) reported that melatonin receptors mediate **contraction of rat cerebral artery**. Male Sprague-Dawley rats (7–8 weeks old) were sacrificed by decapitation and their brains quickly removed and kept in ice-cold Krebs-Ringer buffer aerated with 95% O_2 /5% CO_2 . The posterior communicating artery, between the point at which the internal carotid artery joins and the middle cerebral artery originates, is dissected from the base of the brain, kept in ice-cold Krebs-Ringer buffer, cleaned of excess connective tissue, and transmitted to a vessel chamber filled with cold Krebs-Ringer buffer. Both ends of the artery are mounted on glass cannulas (100 μ m diameter) and secured using 10–0 surgical silk sutures. The cannulas at the proximal and distal ends of the artery are connected to pressure transducers. The artery is perfused with oxygenated Krebs-Ringer buffer using a servo system. The intraluminal pressure of the artery is gradually increased to 60 mmHg by increasing the flow rate of the Krebs-Ringer buffer. The transmural pressure within the artery segment is maintained at 60–65 mmHg, a pressure chosen because it approximates the non-neurogenic pressure exerted on arterial blood vessels in rats. The chamber is superfused with oxygenated Krebs-Ringer buffer maintained at 37°C. The lumen diameter of the artery is continuously measured

using a video electronic analyzer. The preparation is equilibrated for 45–60 min and during this interval it develops the myogenic tone.

Increasing concentrations (10^{-12} – 10^{-6} M) of melatonin are administered through the perfusion buffer, without washout, at regular intervals of 5 min, and the lumen diameter is recorded continuously. Changes in lumen diameter are measured and expressed as percentage contraction.

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N.10.1.7

Melatonin's Effect on Firing Rate of Suprachiasmatic Nucleus Cells

PURPOSE AND RATIONALE

Ying et al. (1996) studied the effects of drugs related to melatonin on neuronal firing activity in the suprachiasmatic nucleus, intergeniculate leaflet, and other brain areas in anesthetized Syrian hamsters. The suprachiasmatic nucleus of the hypothalamus functions as the dominant pacemaker for behavioral and physiological circadian rhythms in mammals (Moore 1983; Meijer and Reitveld 1989). Photic information required for entrainment to lighting cycles reaches the suprachiasmatic nucleus through a direct projection from the retina, and, via an indirect pathway, the geniculohypothalamic tract, originating in the retinorecipient intergeniculate leaflet of the geniculate nuclei (Zhang and Rusak 1989). The firing rates are suppressed by melatonin and its analogs, whereas antagonists reverse this effect (Ying et al. 1996; Liu et al. 1997).

PROCEDURE

Male Syrian hamsters weighing 100–140 g are kept in a photoperiod with 14 h of light beginning at 05:00 a.m. for at least 2 weeks before being used. The animals are anesthetized with 25% urethane (2 g/kg i.p.) and given subcutaneous injections of Robinul (3-hydroxy-1,1-dimethylpyrrolidinium bromide α -cyclopentylmandelate, A.H. Robins; 2%, 0.1–0.2 ml/animal) to reduce congestion of the respiratory tract during anesthesia. Hamsters are mounted in a stereotaxic apparatus and body temperature is maintained at 37°C throughout the experiment. A hole is drilled in the skull overlying the suprachiasmatic nucleus or intergeniculate leaflet region, with the aid of a magnifier. Special care is taken to avoid bleeding caused by damage to the superior sagittal sinus. The eyelids contralateral to the recording site are retracted with sutures and the eye is covered with mineral oil to prevent dehydration after the pupils are dilated with a topical application of 1% atropine sulfate. Electrodes are aimed at the suprachiasmatic nucleus using stereotaxic coordinates (0.2–0.6 mm anterior to the bregma, 0.2–0.35 mm lateral to the midline, and 7.6–7.8 mm ventral to the cortical surface), with the upper incisor bar 2 mm below the interaural line. Electrodes aimed at the hippocampus, dorsal lateral geniculate nucleus and intergeniculate leaflet are inserted 1.4–1.6 mm posterior to the bregma, 3.3–3.8 mm lateral to the midline, and 2.0–4.5 mm ventral to the cortical surface. The animal is maintained in a darkened room except during light pulses or while repositioning the electrodes.

Five-barrel glass micropipettes are prepared (Ying et al. 1993) with the recording barrel filled with fast green (Sigma) at a sub-saturated concentration in 2 M NaCl. One barrel is filled with 2 M NaCl for automatic current balancing. The other barrels are filled with melatonin hydrochloride, or metergoline, or test compound.

For intraperitoneal injections of doses between 0.5 and 10 mg/kg, melatonin and test compounds are dissolved in dimethylsulfoxide (DMSO) and further diluted with saline. For iontophoretic studies, test compounds are used in 5 mM concentrations.

Single-unit extracellular recordings are made from 15:00 to 20:00 hours, overlapping both projected light and dark phases of the daily illumination cycle in the colony room. Contralateral whole-retinal illumination is conveyed to the eye from a tungsten-halogen lamp using fiber optics and a computer-controlled shutter. Photically responsive neurons are identified and recorded during sustained light presentations (typ-

ically 1–2 min). Cells are identified as photically responsive or light sensitive if their firing rates are consistently increased or suppressed by >30% by sustained, whole-retinal illumination.

EVALUATION

A computer program controls data acquisition, light exposures and iontophoretic applications of drugs. Agonist effects are defined as repeatable changes relative to predrug firing rates of >20% at some dose of the drug. In order to compare the effects of melatonin and test compounds, agonist potencies are expressed as ED₅₀ values, the doses required to produce half-maximal effects. The impact of an agonist is expressed as the proportion of the effect of an agonist that is reversed by the co-application of the antagonist; i. e., the difference between the agonist effects in the absence and the presence of the antagonist, divided by the agonist effect alone, and expressed as a percentage.

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N.10.1.8**Melatonin's Effect on Circadian Rhythm****PURPOSE AND RATIONALE**

Melatonin induces phase shifts of circadian rhythms through an action within the suprachiasmatic nucleus of the hypothalamus (Benloucif and Dubocovich 1996; MacArthur et al. 1997; Dubocovich et al. 1998; Delagrange et al. 1999). Competitive melatonin receptor antagonists block phase shifts of circadian rhythms of electrical activity and neuronal firing within the suprachiasmatic nucleus, suggesting that these phys-

iological responses are mediated through activation of melatonin receptors. Dubocovich et al. (1998) tested phase shifts of running activity in C3H/HeN mice induced by melatonin and antagonists. From these data, the authors concluded that selective MT₂ melatonin receptor antagonism is necessary to block melatonin-mediated phase advances of circadian rhythms.

PROCEDURE

Male C3H/HeN mice (5–6 weeks old) are housed in groups of five and maintained in temperature- and humidity-controlled rooms. Food and water are provided ad libitum. Animals are maintained for 2 weeks on a 12-h/12-h light/dark cycle (300 lux at the level of the cage) and are then transferred to constant and complete darkness in individual cages (18 × 30 × 12 cm) equipped with activity wheels. Mice are kept under constant darkness for 3 weeks to stabilize the free-running activity rhythm (TAU = mean duration of the free running circadian period). The animals are treated on 3 days consecutively at circadian time 10 (CT10). (CT12 = onset of activity). CT10 is estimated from the TAU value for each day. Each mouse is treated with vehicle, or melatonin (0.3, 0.9, 3.0, 9.0, or 30 µg melatonin per animal) or the antagonist to be tested at various dosages. Each mouse receives one treatment every 3 weeks and a total of four treatments. All treatments are performed under a dim red light (15 W, Kodak 1A filter) with illuminance of less than 3 lux.

Rhythms of wheel-running activity are measured with a magnetic microswitch, which detects revolutions of the running wheel and is on line with a computer (Benloucif and Dubocovich 1996). Data are collected using the Dataquest III hardware and software package and analyzed with the assistance of TAU software (Mini-mitter, Sunriver, Ore., USA) The exact circadian time of the pulse is determined after treatment from the onset of steady-state activity on the day of the pulse. Aligning the TAU guide by eye over the steady-state activity onsets of free-running activity for 7–10 days before the pulse and from day 4 to 14 after the pulse assesses phase shifts.

EVALUATION

Phase shifts induced by treatment are measured in hours as the difference between the steady-state pre- and post-pulse activity onsets, using the TAU program ruler. Differences between vehicle/melatonin and antagonist/melatonin-treated groups are assessed for each melatonin dose by analysis of variance, followed by pairwise comparison when indicated.

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N.10.1.9

Melatonin's Effect on Neophobia in Mice

PURPOSE AND RATIONALE

Kopp et al. (1999a) studied the effects of melatonin on neophobic responses in different strains of mice. Melatonin counteracted avoidance responses to an unfamiliar environment in BALB/c and C3H/He mice, but not in C5BL/6 mice. In this experimental situation, animals can move freely in simultaneously presented familiar and unfamiliar places (Hughes 1968).

Delagrangé et al. (1999) and Kopp et al. (1999b) tested the effects of a melatonin antagonist on the neophobia-reducing properties of melatonin in BALB/c mice.

PROCEDURE

Male BALB/c mice at an age of 10 weeks are housed in groups of five in standard cages with food and water available ad libitum under controlled conditions at a 12-h light:12-h dark cycle with lights on at 01:00 hours, so that the animals can be observed under dim red light in their active period.

The observation apparatus consists of a rectangular polyvinylchloride box (30 × 20 × 20 cm), covered with Plexiglas and subdivided into six equal square units (10 × 10 × 20 cm), which are all interconnected by small holes located at the floor level. It can be divided in half lengthwise by closing a temporary partition. The apparatus is kept in the mouse room. The experimenter always stands next to the box at the same place. Approximately 24 h before testing each animal is randomly placed in one-half of the apparatus with the temporary partition in place, to be familiarized with it. The floor of this half only is covered with sawdust, and the animal has unlimited access to food and water during 24 h of the familiarization period. At

the end of this period, the temporary partition between the familiar and unfamiliar compartment is removed and the animal then observed for 10 min. Measures are taken of the number of approach responses followed by avoidance reaction toward the unfamiliar places (attempts), the time spent in the unfamiliar compartment (time), the total number of square units entered (locomotion), and the total number of rears made by the animals (rears).

Mice are treated either with saline (controls) or with 1 mg/kg melatonin, or with various doses of the antagonist, or combinations thereof.

EVALUATION

Data are treated with either an analysis of variance (ANOVA) followed by Newman-Keuls a posteriori *t*-test, if groups come from a population with homogeneous standard deviations, or with a Kruskal-Wallis nonparametric ANOVA test followed by Mann-Whitney tests if groups come from a population with heterogeneous standard deviations.

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N.10.2

Melanophore Stimulating Hormone

The early investigations of the pigmentary effects of corticotropin, melanotropin and related peptides derived from the pro-opiomelanocortin family were focused on the control of pigmentary and adrenocortical function (Slominski and Wortsman 2000; Millington 2006). With increasing knowledge and more sophisticated methodology, this role has now been considerably extended (Boehm et al. 2006), including actions on energy balance (Fehm et al. 2004; Butler 2006; Nargund et al. 2006), actions on the cardiovascular system and kidney function (Humphreys 2007), erectile dysfunction (Hadley and Dorr 2006), and even antimi-

icrobial properties (Catania et al. 2006). The pigmentary action of MSH analogs is being commercialized (Hadley and Dorr 2006).

The early studies on synthetic MSH-derived peptide analogs have been considerably extended, with therapeutic applications in view for the control of appetite, and anti-obesic compounds of the future.

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N.10.2.1

Skin Darkening in Whole Amphibia

PURPOSE AND RATIONALE

MSH (melanocyte-stimulating hormone or melanotropin) is also called melanophore expanding hormone, based on the microscopical observation of expansion of melanocytes in amphibia (Landgrebe and Waring 1950, 1962; Sandow et al. 1977; Inouye and Ozsuka 1987). Several amphibia species can be used, such as *Xenopus laevis*, *Rana temporaria* or *R. esculenta*, or *Hyla arborea*. Hypophysectomized animals are more sensitive than intact ones. Numerous corticotropin analogs have MSH activity based on a common peptide core shared with MSH. The structure of two melanocyte-stimulating hormones has been elucidated: α -MSH containing 13 amino acids, and β -MSH containing 18 amino acids.

Hunt (1995) reviewed the role of MSH as a regulator of human melanocyte physiology.

PROCEDURE

The pretest conditions require adaptation for blanching of the skin, e. g., by keeping the animals on a white background prior to the assay. *Xenopus laevis* or *Rana temporaria* are kept in single cages at 16°C under humid conditions. Injections of various doses of the test preparation or the standard are given into the dorsal lymph sac. After 1 h, the webs between the digits of the hind limbs are investigated under a stereomicroscope, the eyepiece of which is fitted with a photoelectric cell, the other being available for microscopic examination. The state of melanophore expansion can be assessed by direct visual examination and recorded in terms of an arbitrary melanophore index, or by modern methods of morphometry.

EVALUATION

For test preparation and standard, dose–response curves can be established and potency ratios calculated.

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N.10.2.2**Assay in Isolated Amphibian Skin****PURPOSE AND RATIONALE**

Melanophores in isolated pieces of pale amphibian skin (from background-adapted animals or those maintained in MSH-free medium) expand within a short period of time after immersion in a MSH-containing buffer. Subsequent immersion of the skin in fresh saline results in contraction of the melanophores (Trendelenburg 1926; Jores 1933; Landgrebe and Waring 1950). Several methods using isolated amphibian skin have been described using either light absorption or light reflection (Landgrebe and Waring 1962). These methods are historical and have been replaced by using cell cultures of melanocytes.

PROCEDURE

For studies on light absorption, the skin of *Rana temporaria* or *R. esculenta* is used. Before sacrificing the donor animal and dissecting the isolated skin, it is important to submit the living animal to varying white and black backgrounds under overhead illumination of a light source of 100 W, so that the melanophores can expand and contract before operation. The areas most suitable for use in this technique are from the thighs. Before using the skin for an actual assay, each piece should be immersed in a sufficiently concentrated MSH solution to expand the melanophores almost but not quite fully. The skin is then washed with saline until the melanophores are fully contracted again and in a suitable condition for the test. This usually takes about 45 min. A piece of skin is mounted in a cell which holds the skin stretched and enables it to be immersed in saline or test solution. It is placed in position on the stage of a binocular microscope. An eyepiece is used for visual observation and a photoelectric cell is attached to the other connected directly to a sensitive galvanometer. Various concentrations of the test preparation or the standard are added. At least six skin pieces are used for each concentration of test preparation or standard. Galvanometer readings and readings of the melanophore index are taken simultaneously every 10–15 min until the maximum response is elicited, usually in 30–45 min.

EVALUATION

Assessment of potency is made from the readings of the melanophore index and from the light absorbed, which is recorded as a percentage of the light originally passing through the skin, and is plotted against the log concentration.

CRITICAL ASSESSMENT OF THE METHOD

The methods were originally developed to standardize extracts with (pigmentary) MSH activity by the use of a bioassay. They are still necessary for evaluating compounds for biological MSH activity since not only natural ACTH but also synthetic peptides, such as β^{1-24} -corticotropin (Schuler et al. 1963) or β^{1-23} -corticotropin-23-amide (Vogel 1965, 1969), possess MSH activity.

MODIFICATIONS OF THE METHOD

Measurement of light reflection by skin pieces, instead of light absorption, was used by Shizume et al. (1954).

In **cell-based assays**, Siegrist and Eberle (1986), Bagutti and Eberle (1993), and Sahm et al. (1993, 1996) used cultured mouse B16 melanoma cells in

a sensitive *in situ* melanoma assay to study the structure–activity relationship of melanocyte-stimulating hormone peptides. B16 cells were seeded at a density of 2500 cells per well in 96-well microtest culture plates. After 24 h the cells were incubated in the presence of serial dilutions of MSH peptides for 3–5 days. The melanin released into the medium of each well was then determined spectrophotometrically at a wavelength of 405 nm using an automatic microplate reader calibrated against synthetic melanin.

Sahm et al. (1994, 1996) measured release of $^3\text{H}_2\text{O}$ into the medium from $[3',5'\text{-}^3\text{H}]\text{L}$ -tyrosine by tyrosinase in B16 mouse melanoma cells after incubation with α -MSH analogs.

MSH assays have also been used to study the factors regulating the release of MSH (**MRF**) or inhibiting the release of MSH (**MIF**) (Kastin et al. 1969; Celis et al. 1971).

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N.10.2.3

Binding to the Melanocortin Receptor

PURPOSE AND RATIONALE

Much of the initial work on the biological effects of hormones has been expanded by using cloned subclasses of receptors for identification of more specific ligand compounds. A family of five MSH receptor subclasses for the melanocortin peptides has been identified (Chhajlani and Wikberg 1992; Chhajlani et al. 1993; Gantz et al. 1993b; Schiöth et al. 1997a, 1988a; Strand 1999). The melanocortin MC₁ receptor is expressed in melanocytes and melanoma cells and binds α -MSH with high affinity. The MC₁ receptor plays an important role in skin and fur pigmentation in a variety of vertebrates (Cone et al. 1996). The melanocortin MC₂ receptor (i. e., the ACTH receptor) has a well-defined function in steroid production in the adrenal gland (Schiöth et al. 1996a). The melanocortin MC₃ receptor is found in the hypothalamus, the brain, and in the placenta, gut tissues and the heart (Gantz et al. 1993a; Desarnaud et al. 1994; Sahm et al. 1994). The melanocortin MC₄ receptor (Schiöth et al. 1996b, 1998b) has been found to affect feeding in rodents and may be important for weight homeostasis (Fan et al. 1997; Huszar et al. 1997). The melanocortin MC₅ receptor is primarily located in various peripheral tissues but has also been found in the brain (Labbé et al. 1994; Fathi et al. 1995).

Schiöth et al. (1997b, 1998a) described binding of synthetic MSH analogs to the human melanocortin receptor subtypes.

Melanocortin-4 receptor antagonists increase food uptake in rats (Kask et al. 1998a, 1998b; Skuladottir et al. 1999).

PROCEDURE

Expression of Receptor Clones

The human melanocortin MC₁ and human melanocortin MC₅ receptors are cloned into the expression vector pRc/CMV (Invitrogen). The human melanocortin MC₃ and human melanocortin MC₄ receptors were cloned into the expression vector

pCMV/neo. For receptor expression COS-1 cells are grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Eighty percent confluent cultures are transfected with the DNA mixed with liposomes in serum-free medium. After transfection, the serum-free medium is replaced by serum-containing medium and the cells are cultivated for 48 h. Cells are then scraped off, centrifuged and used for radioligand binding.

Binding Studies

The transfected cells are washed with binding buffer (Schiöth et al. 1995) and distributed into 96-well non-culture-coated plates, which are centrifuged and the binding buffer is removed. The cells are then immediately incubated in the well plates for 2 h at 37°C with 0.05 ml binding buffer in each well containing a constant concentration of [125I]NDP (= [Nle⁴-D-Phe⁷]α-MSH) and appropriate concentrations of the competing unlabeled ligand. After incubation the cells are washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 M NaOH. Radioactivity is counted by a gamma-counter.

EVALUATION

Data are analyzed by fitting to formulas derived from the law of mass action. The method is generally referred to as computer modeling. K_d values for [125I]NDP are calculated (Schiöth et al. 1995, 1996a). The binding assays are performed in duplicate and repeated three times.

MODIFICATIONS OF THE METHOD

Adan et al. (1994) compared the behavioral effects of melanocortins with binding data on MC₃ and MC₄ receptors.

Sahm et al. (1996) studied receptor binding affinities and biological activities of linear and cyclic melanocortins in B16 murine melanoma cells expressing the native MC₁ receptor.

Quillan and Sadée (1996) searched for peptide ligands that cross-react with melanocortin receptors and found several peptides with previously unrecognized agonistic or antagonistic activity on amphibian and human melanocortin receptors.

Peng et al. (1997) compared the actions of C-terminally modified melanocortin peptides at rodent MC₁ and MC₃ receptors.

Bagutti et al. (1993) recommended [¹¹¹In]DTPA-labeled (where DTPA is diethylenetriaminopentaacetic acid) analogs of α-MSH as ligands for the detection of MSH receptors *in vitro* and *in vivo*.

Erskine-Grout et al. (1996) described functional photoaffinity-labeled, biotinylated and fluorescent probes for the melanoma MC₁ receptor.

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- onists derived from the pro-opiomelanocortin prohormone precursor; and the endogenous antagonists, agouti and agouti-related protein (Gantz and Fong 2003; Cone 2006). This group of regulatory peptides was explored in the context of understanding regulation of body weight development, and finding specific anti-obesic compounds (Bjorbaek and Hollenberg 2002; Della-Fera and Baile 2005; Padwal and Majumdar 2007). As with the leptin research, there are also indications for the central regulation of thermogenesis by melanotropin peptides (Myers 2004; Fan et al. 2005). The subclasses of melanocortin receptors involved in these several functions have been characterized, and the MC₄ receptor is of particular interest (Garcia-Borron et al. 2005; Adan and van Dijk 2006; Adan et al. 2006; Getting 2006). Structure–activity studies on the melanocortin agonists and antagonists have provided further tools for detailed investigation (Cai et al. 2005) by modification of the natural ligands, and by the search for small molecule ligands (Todorovic and Haskell-Luevano 2005). Not surprisingly, the research on body weight regulation has also provided indications for the pathophysiology of cachexia (DeBoer and Marks 2006a, 2006b).
- Interactions of the hypothalamic–pituitary–thyroid axis with the central melanocortin system have been investigated and reviewed (Martin et al. 2006).
- There has also been considerable interest in the regulation of immune responses and inflammation by melanocortin peptides (Maaser et al. 2006), based on the established relationship of α -MSH and inflammation (Eves et al. 2006; Lin and Fisher 2007).

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N.10.3

Melanocortin Peptides

The melanocortin system refers to a set of hormonal, neuropeptidergic, and paracrine signaling pathways that are defined by components that include the five G-protein-coupled melanocortin receptors; peptide ag-

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N.10.4 Relaxin

N.10.4.1 General Considerations

Relaxin is a peptide hormone originally prepared from sow ovaries. The hormone acts on collagen and induces increased flexibility of the pelvic girdle of guinea pigs. In mice, the length of the interpubic ligament is increased. Moreover, relaxin inhibits spontaneous uterine motility. In addition to its effects on the reproductive tract, relaxin exerts hemodynamic effects (Coulson et al. 1996). The actions of relaxin as a pleiotropic hormone were reviewed by Bani (1997).

The therapeutic relevance of relaxin in humans is still open to debate (Schwabe and Büllesbach 1994; Goldsmith et al. 1995).

The primary structure of relaxin is highly homologous to insulin. Unlike insulin, the structure of which is remarkably well conserved among the vertebrates, relaxin sequences can vary by more than 50% between different species (Evans et al. 1993; Layden and Tregear 1996). Despite these large variations, most relaxins have very similar biological activities in ani-

mal test systems, probably because the receptor binding region of the B chain, in contrast to the rest of the molecule, is highly conserved between species.

Relaxin of various species has been synthesized (Büllesbach and Schwabe 1993; Wade et al. 1994).

Synthetic human relaxin has been characterized by high-performance liquid chromatography (Canova-Davis et al. 1990).

Klonisch et al. (1999) determined the nucleic acid sequence of canine **preprorelaxin** using reverse transcription polymerase chain reaction (PCR) and rapid amplification of cDNA ends-PCR. Canine preprorelaxin consists of 534 base pairs encoding a protein of 177 amino acids with a signal peptide of 25 amino acids, a B domain of 35 amino acids, a C domain of 93 amino acids, and an A domain of 24 amino acids.

The **relaxin-like factor (RLF)** is described as a member of the insulin/relaxin/insulin-like growth factor family that is expressed predominantly in the reproductive system, with greatest expression in the Leydig cells of the testis (Pusch et al. 1996; Zarreh-Hoshyari-Khah et al. 1999).

Several **bioassays** have been used for relaxin, such as the pubic symphysis method in guinea pigs and mice, inhibition of uterine motility in rats, and stimulation of interstitial collagenase activity in cultured uterine cervical cells from guinea pigs (see below).

Taylor and Clark (1989, 1992a, 1992b) developed a **reverse hemolytic plaque assay** which allows the quantitative analysis of relaxin secreted by single porcine luteal cells.

Radioimmunoassays for relaxin were described by Sherwood (1979), Jockenhövel et al. (1991), and Steinetz et al. (1996). Lucas et al. (1989) developed an enzyme-linked immunoassay to study human relaxin in human pregnancy and in pregnant rhesus monkeys.

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N.10.4.2**Relaxin Bioassay by Pubic Symphysis Method in Guinea Pigs****PURPOSE AND RATIONALE**

Experimental relaxation of the pubic symphysis of the spayed guinea pig was described as early as 1929 by Hisaw. The relaxation of the symphysis after relaxin administration is determined by manual palpation.

PROCEDURE

Virgin female guinea pigs of mixed strains weighing 300–400 g are used. Prior to the assay, the animals have to be primed with estrogen and relaxin. Estrogen priming consists of one subcutaneous injection per week of 5 µg of estradiol cyclopentylpropionate in 0.1 ml of sesame oil. Relaxin priming is accomplished by administering 20 µg of relaxin standard in 1 ml saline subcutaneously once a week on day 5 after the estrogen injection.

Six hours after relaxin administration the symphysis of the animal is palpated. The animal is held head down, ventral side away, between the thighs of the seated observer. The sciatic crests and symphysis pubis are firmly grasped between the thumbs and forefingers so that the two halves of the pelvis may be moved back and forth alternately. If the pubic symphysis is rigid at this time, the estrogen and relaxin priming are continued weekly until marked mobility of the symphysis is observed. The increased flexibility is transient: the peak response occurs at 6 h and subsides 12–24 h after injection. Mobility responses are estimated subjectively and scored on an arbitrary scale of 0–6. “Zero” indicates no detectable flexibility of the pubic symphysis, whereas “6” represents extreme softening. Scoring should be performed by the same investigator throughout a study.

One week after an animal has responded positively to 20 µg of relaxin standard, it is added to the assay colony. Before assay time, all eligible animals are mixed and divided into groups of 10–20 each. On the day of the experiment, all animals are palpated before injection. Only those with no symphyseal movement are used. Two doses of test preparation and two doses of standard are injected subcutaneously to different groups. Six hours later, two operators palpate and score each animal.

EVALUATION

The scores are averaged and a median score calculated. The activity of an unknown preparation is determined by comparison with the dose–response curve of concomitantly administered relaxin standard.

MODIFICATIONS OF THE METHOD

Steinetz and Lust (1994) reported that the relaxin-induced pubic symphyseal relaxation in guinea pigs is inhibited by treatment with glycosaminoglycan polysulfates or pentosan polysulfate. The authors recommended the guinea pig symphysis assay for relaxin as a novel rapid screening test for compounds with potential chondroprotective activity.

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N.10.4.3**Relaxin Bioassay in Mice****PURPOSE AND RATIONALE**

An increase in length of the interpubic ligament in mice can be used as a bioassay for relaxin. Other methods used include X-ray measurement of pubic separation (Hall 1948; Dorfman et al. 1953) and direct measurement of interpubic ligament (Kroc et al. 1959; Steinetz et al. 1960, 1969a, 1969b).

PROCEDURE

Virgin female mice (e.g., NMRI strain) weighing 18–20 g are used for the assay. Twenty mice are employed for each of three dose levels of standard and test preparation. On day zero each mouse is primed with a single subcutaneous injection of 5 µg estradiol cyclopentylpropionate in 0.1 ml sesame oil. On day 7, the test preparation and the standard are injected subcutaneously. At 18–24 h later, the mice are sacrificed, the abdominal cavities are opened, and uteri are examined for evidence of estrogen priming. Mice exhibiting threadlike uteri are discarded. The anal and vulval areas are then cut away with scissors, and the upper half of the body is cut off to prevent subsequent bleeding at the pubic symphysis. The bony birth canal is freed of skin, vagina, and rectum, and fascia are cleaned off the symphysis pubis. The pelvis is placed on a binocular microscope fitted with a calibrated ocular micrometer. A transilluminating device consisting of a U-shaped lucite rod is affixed to the microscope. The tip of the rod is beveled to direct light vertically through the exposed pubic ligament. The feet of the carcass are grasped between the thumb and index finger, applying a slight lateral traction.

The shortest distance between the edges of the pubes is measured, using the ocular micrometer. Micrometer readings are then converted to millimeters.

EVALUATION

Mean values of length of interpubic ligament, expressed in millimeters, are plotted versus logarithm of dose. From dose–response curves activity ratios with confidence limits versus the standard are calculated.

MODIFICATIONS OF THE METHOD

Bullesbach and Schwabe (1996) tested rat relaxin and synthetic analogs in the mouse symphysis pubis assay for structure–activity relationships.

Samuel et al. (1998) studied the effects of relaxin, pregnancy and parturition on collagen metabolism in the rat pubic symphysis. During pregnancy and particularly during birth, there was a significant reduction of tissue wet and dry weight, which coincided with an increase in water content and a significant reduction of overall collagen content.

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N.10.4.4**Inhibition of Uterine Motility****PURPOSE AND RATIONALE**

This is one of the historical assays based on a nonspecific biological response. Its application and reliability depend on the state of purification of relaxin preparations. Relaxin specifically inhibits the spontaneous motility of estrogen-dominated uterus *in vivo* and *in*

vitro (Downing and Hollingsworth 1993). Wiquist and Paul (1958) proposed a relaxin assay based on inhibition of motility of the rat uterus *in vitro* (Steinetz et al. 1969).

PROCEDURE

The donors (female Sprague-Dawley or Wistar rats weighing 150–180 g) are ovariectomized and primed with 2 µg estradiol daily for 3 days. On day 4, uterine horns are removed, bisected, and suspended in Locke's solution gassed with 1% CO₂ in O₂ at a temperature of 37.5 ± 0.5°C. The contractions are recorded using a Statham transducer and a polygraph. A symmetrical 4-point assay is adopted with twofold dose increments of standard and test preparation. Responses consisting of slowing down the contraction frequency up to total suppression of the contractions are classified visually and assigned score values of 1–3. All four test doses (two doses each of standard and test preparation) are run simultaneously on the four uterine segments obtained from each rat.

EVALUATION

Mean values of scores are calculated for each dose. Potency ratios with confidence limits are calculated from the 2+2-point assay.

MODIFICATIONS OF THE METHOD

Felton et al. (1953) described a test for relaxin activity using the inhibition of uterine contraction in anesthetized guinea pigs *in vivo*.

Inhibition of spontaneous and prostaglandin-driven myometrial activity by relaxin in anesthetized rats was reported by Porter et al. (1979).

Downing and Sherwood (1985) studied the influence of relaxin on uterus contractility and on cervical distensibility in different stages of pregnancy in the rat.

Del Angel Meza et al. (1991) measured the effect of relaxin on uterine and ileum tissue of rats *in vivo* and *in vitro*.

Vu et al. (1993) tested the activity of a recombinant prorelaxin in an *in vitro* bioassay in CHO cells. Human uterine endometrial cells were treated with various dilutions of conditioned medium and the amounts of intracellular cAMP produced were determined by radioimmunoassay.

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N.10.4.5

Relaxin Assay by Interstitial Collagenase Activity in Cultured Uterine Cervical Cells

PURPOSE AND RATIONALE

As an example of the changing approach in assay methods, Mushayandebvu and Rajabi (1995) provided evidence for a biological response suitable for *in vitro* assay. Relaxin is involved in cervical dilatation by stimulating interstitial collagenase, a key enzyme involved in this process. Human recombinant relaxin induces a dose-dependent increase of collagenase activity in cultured guinea pig cervical cells.

PROCEDURE

Abdominal hysterectomy is performed in anesthetized female Hartley guinea pigs under aseptic conditions. The cervix is excised and washed three times in Hank's balanced salt solution (HBSS) containing penicillin G sodium (100 U/ml), streptomycin sulfate 100 µg/ml, and amphotericin B (1.25 µg/ml). The cervix is cut into 2- to 4-mm pieces and digested in Dulbecco's modified Eagle's medium (DMEM) containing bacterial collagenase type A1 (0.5 mg/ml) and DNase type 1 (0.05 mg/ml) at 37°C. The digested mixture is filtered once every 15–30 min through a nylon monofilament with a pore size of 400 µm. Following each filtration, separated cells are collected by centrifugation at 365 g for 10 min at room temperature followed by a resuspension in DMEM containing 10% heat-inactivated fetal calf serum (FCS). Cells are washed twice with HBSS; this is followed by resuspension in DMEM containing 10%

penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (1.25 µg/ml). The unfiltered material is redigested and the process is repeated until the cervical tissue is completely digested.

Cells are plated in 24-well plates at 1×10^5 cells per well in 500 µl of medium containing 10% FCS and antibiotics and then are incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. After 4 days all viable cells are adhered. Culture medium and unattached cells are removed and fresh medium is added. At confluency (5–7 days), culture medium is removed, cells are washed once with HBSS, and serum-free DMEM containing antibiotics is added. The cells are treated with recombinant human relaxin (1–1000 ng/ml) at 0, 24, and 48 h. Culture media are collected at 96 h from initial treatment with the appropriate controls and frozen immediately at –20°C until assayed for collagenase activity (Dean and Woessner 1985; Rajabi et al. 1988, 1991).

Enzyme samples [up to 100 µl in assay buffer: 50 mM Tris HCl (pH 7.5), 0.2 M NaCl, 10 mM CaCl₂, 0.02% sodium azide, 0.035% Brij-35] are incubated with 10 µl of [³H]telopeptide-free collagen substrate [specific activity 3.1×10^6 cpm/mg collagen at 2.24 mg/ml, in 50 mM Tris HCl (pH 7.6), 0.3 M NaCl] in 1.5-ml microfuge tubes. Aminophenylmercuric acetate (0.5 mM) is used to activate procollagenase; 1,10-phenanthroline (1 mM) is added to inhibit collagenase and serves as blank control for nonspecific collagenolysis. After 18–48 h of incubation at 29°C, the reaction is terminated by addition of EDTA to a final concentration of 40 mM. Collagenase cleavage products are further digested by trypsin and chymotrypsin in the presence of BSA (10 mg/ml assay buffer) for 2 h at 29°C. The undigested [³H]telopeptide-free substrate is precipitated in 10% trichloroacetic acid at 0°C for 30 min followed by centrifugation at 18,000 g at 4°C for 30 min. A 100-µl aliquot of the supernatant is mixed in 5 ml Aquasol scintillation fluid and counted in a liquid scintillation counter.

EVALUATION

The percentage digestion is calculated as the total cpm in the supernatant minus cpm in the 1,10-phenanthroline blanks divided by original counts in the [³H]telopeptide-free substrate (in 100 µl of 10% trichloroacetic acid in assay buffer) $\times 100$. One milliunit of collagenase is defined as the amount of collagenase that digests 1 ng of collagen in 1 min at 29°C.

All results are presented as the mean \pm SEM of three separate experiments. Statistical analysis is performed

by Student's *t*-test for paired observations and by one-way ANOVA for multiple observations.

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N.10.4.6

Relaxin Receptor Binding

PURPOSE AND RATIONALE

Using a monocomponent, high specific activity, carrier-free porcine relaxin tracer [¹²⁵I], Yang et al. (1992) demonstrated relaxin receptors in the symphysis pubis, uterus, and ovary of mice. A linear Scatchard plot suggested the presence of only one kind of receptor and a dissociation constant of 5×10^{-10} M.

PROCEDURE

Crude membranes are prepared from uterine horns of estrogen-primed mice. The tissues are homogenized three times for 10 s with a Polytron homogenizer. The homogenate is centrifuged at 700 g for 10 min, and the supernatants are recovered and recentrifuged twice at the same speed. Thereafter, the supernatants are centrifuged at 10,000 g for 30 min. The crude membrane pellets are washed with HEPES buffer, centrifuged again, the resuspended in 200–500 µl water, and aliquots are removed for Lowry protein estimates.

The crude membrane suspension is diluted to 3 mg/ml protein with binding buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 1.6 mM CaCl₂, 0.025 mM MgCl₂, 1.5 mM MnCl₂, 1% BSA, 2.8 mM glucose, 0.2 mM phenylmethylsulfonyl fluoride, 80 mg/l soybean trypsin inhibitor, pH 7.5). The assay is initiated by adding this to 120 µl crude membranes (300 µg protein) with and without 0.2 µg unlabeled relaxin. At the end of the incubation period, 1 ml HEPES buffer, containing 1% BSA, is added, and bound and free relaxin are separated by three successive washings and centrifugations.

EVALUATION

The tubes are analyzed for radioactivity in a Mini-gamma 400 (LKB, Rockville, Md., USA). Duplicates are run for each experimental point, and experiments are repeated several times.

MODIFICATIONS OF THE METHOD

The control of relaxin secretion and relaxin receptors by relaxin was studied by Bryant-Greenwood et al. (1982).

Relaxin receptors in the myometrium of rats and pigs were studied by Mercado-Simmen et al. (1982a, 1982b).

Fluoresceinylthiocarbamyl relaxin was prepared by Segaloff and Gabbard (1982) for the demonstration of relaxin receptors.

Experiments by Büllsbach and Schwabe (1988) suggested a unique site for the interaction of relaxin with its uterine and symphyseal receptors.

Using a ^{32}P -labeled human relaxin, Osheroff and Phillips (1991) localized relaxin binding sites in rat uterus, cervix, and brain.

The receptor binding site of human relaxin II was studied by Büllsbach et al. (1992).

Min and Sherwood (1996) identified specific cell types that contain relaxin receptors in various organs of pregnant pigs.

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N.10.5

Calcitonin Gene-Related Peptide

N.10.5.1

General Considerations

In relation to the established traditional hormone definition, many new polypeptides have been identified by molecular biology and characterized by receptor binding and *in vitro* systems, while their definitive function is not yet assigned. Calcitonin-gene-related peptide (CGRP) is an example of this rapid development and change in approach by applying new methods. CGRP is a 37-amino-acid neuropeptide which was first identified as a product of the calcitonin gene by alternative splicing (Amara et al. 1982). This original peptide is referred to as α -CGRP; a second gene, unrelated to calcitonin, produces β -CGRP (Amara et al. 1985). These two forms are closely related in both rat and humans, and are widely distributed throughout most parts of the nervous system. CGRP shares about 50% identity with another 37-amino-acid peptide, amylin (Rink et al. 1993). CGRP shows weak but significant homology with adrenomedullin, a 52-amino-acid peptide (Kitamura et al. 1993). Several forms of CGRP have been sequenced: α - and β -CGRP from rat and humans, and single variants from sheep, pig, chick, salmon, and the laughing frog, *Rana ridibunda* (Poyner 1997). The calcitonin/CGRP gene is expressed in specific cell types of both the endocrine and nervous systems. The gene is alternatively spliced to yield mRNA encoding calcitonin in thyroid C-cells or the neuropeptide CGRP in a subset of central and peripheral neurons (Amara et al. 1982; Morris et al. 1984; Born and Fischer 1993). The rat as well as the human calcitonin/CGRP gene consists of six exons. The calcitonin mRNA contains exons 1–4 with a poly(A) tail at exon 4 whereas CGRP α includes exons 1, 2, 3, 5, and 6 with a poly(A) tail at exon 6 (Van Rossum et al. 1997).

α - and β -CGRP display several biological activities, including peripheral and cerebral vasodilatation, a blood-pressure-lowering effect, cardiac acceleration, regulation of calcium metabolism, reduction of intestinal motility, regulation of glucose metabolism, stimulation of pancreatic enzyme secretion, diminution of appetite, reduction of growth hormone release, influence on inflammation and nociception, and inhibition of interleukin-2 production (Poyner 1992, 1997; Wang et al. 1992; Wimalawansa 1996).

Nuki et al. (1994) compared the vasodilatory activity of chicken calcitonin gene-related peptide with human α -CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats (see Sect. A.8.2.5).

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologs, and antagonists in rat isolated pulmonary artery (see Sect. A.1.2.1) and rat deferens (see Sect. A.1.2.3).

Tomobe et al. (1998) found that the vasodilatation in isolated superior mesenteric arteries by calcitonin gene-related peptide was significantly larger in spontaneously hypertensive rats than in normal Wistar-Kyoto rats.

In order to elucidate the mechanism of endogenous CGRP release in peripheral vasodilatation, Brain et al. (1993) used a multiple site ^{133}Xe clearance technique.

Raddino et al. (1997) studied the mechanism of action of human calcitonin gene-related peptide in rabbit heart and human mammary arteries.

Champion et al. (1997) analyzed the responses of human synthetic adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat (see Sect. A.8.2.1).

Castellucci et al. (1993) investigated the vasodilator activity of CGRP in the rat isolated and perfused kidney.

McMurdo et al. (1997) investigated the effect of the calcitonin gene-related peptide receptor antagonist CGRP⁸⁻³⁷ on blood flow in the knee joint of anesthetized rats. Synovial blood flow was measured in both exposed and intact skin-covered knees by Laser Doppler perfusion imaging.

Sakai et al. (1998) found a synergism of calcitonin gene-related peptide with the blood-pressure-lowering effect of adenosine.

Preibisz (1993) reported beneficial effects of CGRP infusions in patients with congestive heart failure and in subjects with neurological deficits after surgical treatment of subarachnoid hemorrhage.

Cadieux et al. (1999) described bronchoprotector properties of calcitonin gene-related peptide in guinea pig and human airways. Calcitonin gene-related peptide inhibited substance-P-induced bronchoconstriction *in vivo* and *in vitro*.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α -calcitonin gene-related peptide to stimulate amylase secretion from guinea pig pancreatic acini and to relax precontracted mesenteric arteries (see Sect. A.1.2.9).

Meini et al. (1995) investigated the propagation of impulses in the guinea pig ureter and its blockade by calcitonin gene-related peptide. Furthermore, Maggi et al. (1995) studied the mechanisms of the inhibitory effect exerted by calcitonin gene-related peptide on the spontaneous activity of the guinea pig isolated renal pelvis.

Protective effects of calcitonin gene-related peptide in different experimental models of gastric ulcers (reserpine-induced gastric lesions, ethanol-induced gastric lesions, gastric damage and acid secretion in pylorus-ligated rats) were reported by Clementi et al. (1993).

The role of nitric oxide in the anti-ulcer activity of calcitonin gene-related peptide was investigated by Clementi et al. (1994a).

Evangelista and Renzi (1997) investigated the protective role of endogenous and exogenous calcitonin gene-related peptide in water immersion stress-induced gastric ulcers in rats.

Li et al. (1997) determined the ability of analogs of human α -calcitonin gene-related peptide to stimulate amylase secretion from guinea pig pancreatic acini (see Sect. J.7.0.10) and to relax isolated porcine coronary arteries precontracted with 20 mM KCl (see Sect. A.3.1.3).

Calcitonin gene-related peptide acutely augments the contractile response of skeletal muscle to both direct and indirect stimulation as studied in the isolated rat diaphragm by Fleming et al. (1993).

Dumont et al. (1997) used the isolated guinea pig heart (see Sect. A.3.1.2) and the isolated rat vas deferens (see Sect. A.1.2.3) for *in vitro* bioassays of CGRP agonists and antagonists.

Poyner et al. (1999) found a concentration-dependent inhibition of the electrically stimulated twitch response of guinea pig vas deferens by calcitonin gene-related peptide, amylin and adrenomedullin (see Sect. A.1.2.3).

The role of calcitonin gene-related peptide in the protection of capsaicin-induced gastric mucosal hyperemia in rats was studied by Merchant et al. (1994).

Clementi et al. (1994b) studied the anti-inflammatory activity of calcitonin gene-related peptide in cutaneous inflammation induced by Croton oil, arachidonic acid, tetradecanoylphorbol acetate or cantharidin.

Schaible (1996) investigated the role of tachykinins and calcitonin gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-evoked hyperexcitability in spinal cord neurons.

The development of tolerance to spinal morphine analgesia in rats was prevented by a calcitonin gene-related peptide receptor antagonist (Menard et al. 1996).

Lutz et al. (1997) investigated the anorectic effects of CGRP and amylin in rats chronically cannulated in the lateral brain ventricle.

Specific calcitonin gene-related peptide receptors were characterized in hamster pancreatic cells (Barakat et al. 1993).

In doses of 25–200 µg/kg i.p., CGRP decreased food intake in mice, suggesting a role for CGRP as a satiety factor (Morley et al. 1996).

CGRP inhibits insulin-stimulated glycogen synthesis in rat skeletal muscle (Leighton and Cooper 1988).

Chatzipantelli et al. (1996) described the lipolytic actions of calcitonin gene-related peptide.

Howitt and Poyner (1997) determined the effects of a series of agonists and antagonists on the calcitonin gene-related peptide receptor of cultured rat L6 skeletal myocytes.

Kurz et al. (1995) studied the receptors of calcitonin gene-related peptide in the rat thymus and suggested that CGRP is a paracrine thymic mediator that may influence the differentiation, maturation, and proliferation of thymocytes.

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N.10.5.2

Receptor Binding of CGRP

PURPOSE AND RATIONALE

Calcitonin gene-related peptide receptors are localized in many tissues, such as brain, heart, spleen, blood vessels, liver, lung, and kidney (Born and Fischer 1993; Muff et al. 1995). They are abundant in the brain, and the pattern is similar in the rat, pig, cow, sheep, and humans (Wimalawansa and El-Kholy 1993, 1996).

Multiple CGRP receptors (subclasses) have been observed: based on pharmacological properties they are divided into at least two subtypes and denoted as CGRP₁ and CGRP₂ (Dennis et al. 1989, 1991; Quirion 1992; Poyner 1997). CGRP-(8-37), which lacks seven terminal amino acid residues, is a selective antagonist of CGRP₁ receptors, whereas the linear analog of CGRP, diacetoamidomethyl cysteine CGRP (Cys[ACM_{2,7}]CGRP), is a selective agonist of CGRP₂ receptors.

An atypical CGRP subtype has been reported (Dennis et al. 1991; Van Rossum 1997).

A sensitive and specific radioreceptor assay for calcitonin gene-related peptide was described by Wimalawansa (1989).

PROCEDURE

For membrane preparation, tissue samples, e. g., dissected brain areas from rats, are placed in 10 vols of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose, 0.5 mM dithiothreitol, and 5 mM EDTA. After centrifugation of the homogenate at 100 g for 10 min, the resultant pellet is rehomogenized with 5 vols of fresh buffer and recentrifuged. The pellet is discarded and the supernatants are pooled and recentrifuged at 30,000 g for 45 min at 4°C. The resulting crude membrane pellet is resuspended in 5 vols of assay buffer (50 mM Tris-HCl, 10 mM KCl, 3 mM sodium azide, and 200 IU/ml aprotinin) and recentrifuged at 30,000 g for a further 45 min. This procedure is repeated twice with fresh assay buffer. Protein concentrations are determined and adjusted to 1–2 mg/ml. The samples are frozen on dry ice and stored at –70°C until radioligand binding studies.

For radioligand binding studies, [¹²⁵I]CGRP (25 pM, ~ 5 fmol containing ~ 20,000 cpm/tube) is incubated with various membrane preparations (150–200 µg/ml membrane protein in an incubation medium of 200 µl) in the presence of 200 IU/ml aprotinin and 0.2% heat-inactivated BSA at 4°C for 120 min in polypropylene microcentrifuge tubes in a shaking water bath. At the end of the incubation period, 700 µl of chilled assay buffer is added and immediately centrifuged at 11,000 g for 2 min in a refrigerated microcentrifuge. The supernatant is discarded and the resulting membrane pellet is rewashed and the radioactivity remaining in the pellet is counted.

EVALUATION

Specific binding is calculated by subtracting the [¹²⁵I]CGRP binding in the presence of unlabeled

CGRP. Results are expressed as fmol of [125 I]CGRP bound/mg membrane protein.

MODIFICATIONS OF THE METHOD

Van Rossum et al. (1994) described the binding profile of a selective calcitonin gene-related peptide receptor antagonist ligand, [125 I-Tyr]hCGRP₈₋₃₇, in rat brain and peripheral tissues.

Muff et al. (1992) reported that the calcitonin gene-related peptide receptor in a human neuroblastoma cell line (SK-N-MC) is clearly different from the calcitonin receptor in a human breast carcinoma cell line (T47D).

Aiyar et al. (1996) described a cDNA encoding the calcitonin gene-related peptide type 1 receptor. Stable expression in human embryonic kidney 293 (HEK 293) cells produced specific high-affinity binding sites for CGRP that displayed pharmacological and functional properties very similar to those of the native human CGRP₁ receptor.

Poyner et al. (1999) characterized the receptors for calcitonin gene-related peptide and adrenomedullin on the guinea pig vas deferens.

Juaneda et al. (2000) reviewed the molecular pharmacology of CGRP and related peptide receptor subtypes.

Schindler and Doods (2002) described binding properties of the nonpeptide CGRP receptor antagonist radioligand, [3 H]BIBN4096BS.

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N.10.6

Inhibin

N.10.6.1

General Considerations

Inhibin is a gonadal dimeric glycoprotein that has an inhibitory effect on the secretion of follicle-stimulating hormone by the pituitary gland. The existence of a nonsteroidal gonadal hormone was described by McGullagh as early as 1932. The hormone was isolated and purified from human seminal plasma, ram rete testis fluid, and from bovine follicular fluid (Franchimont et al. 1979, 1989; Robertson et al. 1986; Vale et al. 1986; De Kretser and Robertson 1989). After the determination of the full structure of bovine (Forage et al. 1986) and human (Mason et al. 1986; Stewart et al. 1986; Tierney et al. 1990) inhibin, it was found that inhibin shares structural homology with a family of glycoproteins which includes Mullarian inhibiting substance, transforming growth factor- β , follistatin, activin, and bone morphogenic proteins (Robertson 1991; Moore et al. 1994). Inhibin is a disulfide-linked dimer of an α -subunit and a structurally related β -subunit, either β_A or β_B (Robertson et al. 1992). Inhibin A and inhibin B are related dimeric

protein hormones and endocrine regulators of the reproductive axis which show differing patterns during the period of follicular development (Woodruff et al. 1996).

Clinically, inhibin determination may have uses in reproductive medicine and oncology; the diagnosis of some forms of cancer including granulosa cell tumors, cystadenocarcinoma of the ovary and hydatidiform mole and in the physiology and pathology of pregnancy including placental function (Halvorson and DeCherney 1996).

An International Standard for Porcine Inhibin was described by Gaines Das et al. (1992). An International Standard for human recombinant inhibin was established by Rose and Gaines Das (1996).

Tio et al. (1994) purified gonadotropin surge-inhibiting factor (GnSIF), a monomeric polypeptide that shares some biological activities with inhibin, from Sertoli cell-enriched medium.

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N.10.6.2

In Vitro Bioassay for Inhibin

PURPOSE AND RATIONALE

Several modifications of *in vitro* bioassays for inhibin have been used to establish the International Standard for porcine inhibin (Gaines Das et al. 1992) and for human recombinant inhibin (Rose and Gaines Das 1996). Mason et al. (1996) used rat anterior pituitary cells for characterization and determination of the biological activities of noncleavable high-molecular-weight forms of inhibin A and activin A.

PROCEDURE

Anterior pituitary cells are prepared from adult male rats and added to 48-well cluster plates at an initial density of 75,000 cell per 0.4 ml of medium per well. Cultures are preincubated in DMEM-Ham's F12 (1:1) medium with bicarbonate supplemented with nonessential amino acids, antibiotics (100 U/ml penicillin, 100 pg/ml streptomycin, and 250 ng/ml fungizone), and 10% charcoal-stripped FBS. Two days later, the cells are washed, and the medium is replaced with serum-free medium supplemented with antibiotics and recombinant human inhibin A (20–100 pM) as standard. Cultures are incubated with test samples for 65–72 h, after which the samples of medium are collected and stored at -20°C until assayed for follicle-stimulating hormone (FSH) by radioimmunoassay (RIA) using goat second antibody precipitation with

the following reagents (NIDDK): rat FSH-RP-2, rat FSH-I-8 iodinated using Iodogen, and anti-rat FSH-S-11 primary antiserum. Each sample for bioassay is tested at least twice in independent cell cultures to confirm the observations.

EVALUATION

Characteristics of the concentration–response curve, including the maximum effect and medium inhibitory concentration (IC_{50}), are computed using the Allfit program (Munson and Rodbard 1980).

MODIFICATIONS OF THE METHOD

Robertson et al. (1991) studied *in vivo* the FSH-suppressing activity of human recombinant inhibin A in the serum of male and female rats.

Wreford et al. (1994) studied the age dependence of gonadotropin-suppressing activity of human recombinant inhibin in the serum of male rats.

Simpson et al. (1992) induced bilateral cryptorchidism in adult male Sprague Dawley rats under ether anesthesia by cutting the gubernaculum of both testes, translocating the testes to the abdominal cavity and ligating the inguinal canal to prevent redescend of the testes into the scrotum. After 28 days, Sertoli cell cultures from these rats were prepared and inhibin secretion in response to follicle-stimulating hormone (FSH) was measured.

Brown et al. (1991) investigated the effects of inhibin-rich porcine follicular fluid administration on serum bioactive and immunoreactive FSH concentrations and compensatory testosterone secretion in hemicastrated adult rats.

Hertan et al. (1999) used primary cultures of ovine anterior pituitary cells for bioassays of inhibin and identified high-affinity binding sites for inhibin using iodinated recombinant human 31-kDa inhibin.

Jakubowiak et al. (1989) found similar effects of inhibin and cycloheximide on gonadotropin release in superfused rat pituitary cell cultures.

Demura et al. (1996) studied the levels of inhibin α , β_A and β_B subunit mRNAs by a quantitative reverse transcription-polymerase chain reaction and the changes in their levels caused by adding inhibin α , β_A and β_B subunit mRNA antisense oligonucleotides and inhibin A, activin A or gonadotropin-releasing hormone (GnRH) to cultured rat anterior pituitary cells.

Robertson et al. (1996) investigated the specificity of several immunoassay methods in terms of their ability to detect the range of inhibin forms found in plasma and their relationship to bioactivity.

A commercially available enzyme-linked immunosorbent assay (ELISA) for inhibin A (Serotec, Oxford) was used by several authors (Wenstrom et al. 1997; Blumenfeld et al. 1998; Wallace et al. 1998). Magoffin and Jakimiuk (1998) used specific and sensitive two-site enzyme-linked immunosorbent assays for determination of inhibin A, inhibin B, and activin A.

An *in vitro* method has been developed by Allenby et al. (1991) for culturing isolated seminiferous tubules from adult rats for 1–3 days and optimized on the basis of the secretion of immunoreactive inhibin under basal conditions and after maximal stimulation with rat FSH or dibutyl cAMP. Inhibin was measured using a double-antibody radioimmunoassay based on an antibody generated in a sheep to the 1–26 sequence (plus glycine²⁷tyrosine²⁸) of the N-terminus of the α -subunit of porcine 32-kDa inhibin. The effect of three known testicular toxicants (meta-dinitrobenzene, nitrobenzene, and methoxy acetic acid) on these cultures was assessed.

Knight et al. (1991) described the development of a two-site immunoradiometric assay for dimeric inhibin using antibodies against chemically synthesized fragments of the α and β subunit. Knight and Muttukrishna (1994) described the measurement of dimeric inhibin using a modified two-site immunoradiometric assay specific for oxidized (Met O) inhibin.

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N.10.7

Activin

N.10.7.1

General Considerations

Activin is a pluripotent growth factor that was originally isolated based on its ability to stimulate follicle-stimulating hormone (FSH), but is now known to have other important roles during development, erythropoiesis, inflammation, and wound healing (De Paolo 1997). Three types of activin, formed by dimerization of two inhibin- β subunits β_A and β_B , are termed activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$) and activin B ($\beta_B\beta_B$) (Ling et al. 1986; Vale et al. 1986; Mason et al.

1989; Nakamura et al. 1992). In addition, a third β -subunit (β_C) has been identified (Hötten et al. 1995; Loveland et al. 1996). Lee et al. (1989) suggested that, in the testis, the Leydig cells secrete activin and the Sertoli cells produce inhibin, or a combination of both. As members of the transforming growth factor beta (TGF- β) family, the activins are involved in a diverse range of physiological processes, including the regulation of FSH biosynthesis and secretion (Mason 1988; MacConnell et al. 1999), steps in embryonic development (Thomsen et al. 1990), spermatogonial mitosis (Mather et al. 1990) and erythroid differentiation (Eto et al. 1987). Activin acts via a family of activin receptor subunits that includes one type I (Act RI or ALK-2) and two homologous type II (IIA and IIB) subunits (Dalkin et al. 1996; Hashimoto et al. 1998).

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N.10.7.2

In Vitro Bioassay for Activin

PURPOSE AND RATIONALE

In vitro bioassays for activin have been developed using the production of follicle-stimulating hormone (FSH) from cultured pituitary cells (Robertson et al. 1992), the accumulation of hemoglobin in K562 erythroleukaemia cells (Schwall and Lai 1991), and the ability to induce mesoderm tissue in animal cap explants of *Xenopus* (De Winter et al. 1992). One of the sites of production of activin A is the bone marrow (Shao et al. 1992; Yamashita et al. 1992; Uchimaruru et al. 1995). Brosh et al. (1995) found that the mouse plasmacytoma cell line MPC-11 (Laskov and Scharff 1970) was exquisitely sensitive to inhibition by activin A without being influenced by a variety of other cytokines and growth factors. On this basis, Phillips et al. (1999) evaluated the MPC-11 cell line as the basis for an *in vitro* bioassay for activin.

PROCEDURE

MPC-11 plasmacytoma cells are cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 24 mM bicarbonate, L-glutamine, nonessential amino acids and Pen-strep in a 95% air/5% CO₂ atmosphere at 37°C, and passaged at 1 × 10⁶ cells/25 cm² flask every second day. For experiments, 100 µl cell suspension is added to 96-well plates at a density of 1000 viable cells/well in culture medium containing 25 µM β-mercaptoethanol. Activin, other test reagents and sera (100 µl/well) are added to the cultures diluted in phosphate buffer (pH 7.4) containing 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.14 M NaCl, 0.01% BSA and Pen-strep. Cells are cultured in the presence of test reagents for 2 days; on the third day 25 µl [³H]thymidine (0.25 µCi/25 µl, 6.7 Ci/mmol) is added to each well and

24 h later the cells harvested and thymidine incorporation assessed. Proliferation of MPC-11 cells, as measured by thymidine incorporation, is inhibited by increasing doses of activin A or activin B, whereas follistatin, inhibin A, LH, and interleukin-1β are ineffective.

EVALUATION

All test preparations, including activin standards and sera, are assayed in quadruplicate, and experiments are repeated at least twice. Relative bioactivity and parallelism are assessed using parallel-line bioassay statistics.

MODIFICATIONS OF THE METHOD

Mesoderm induction assays in *Xenopus* were used by Wuytens et al. (1999). *Xenopus* embryos were obtained by *in vitro* fertilization. They were maintained in 10% Normal Amphibian Medium and staged according to Nieuwkoop and Faber (1967). Animal pole regions were dissected from mid-blastula (stage 8) embryos and cultured in 75% Normal Amphibian Medium containing 0.1% bovine serum albumin and wild-type or mutant activin (2.5 ng/ml). A preliminary assessment of mesoderm induction was based on the elongation of the animal caps. Animal pole regions were then frozen on dry ice, and expression of the mesoderm-specific gene Brachyury (Xbra) was assessed by RNase protection analysis.

LaPolt et al. (1989) examined the effects of purified porcine activin on inhibin secretion and messenger RNA levels in cultured granulosa cells obtained from immature, estrogen-treated rats. Western blot analyses performed with affinity-purified antisera to inhibin α- and β_A-subunits revealed that treatment with either FSH or activin increased the secretion of inhibin αβ dimer, with a further increase after co-treatment.

Attardi and Miklos (1990) examined the effect of purified recombinant human activin A on steady-state levels of mRNAs for the gonadotropin subunits in pituitary cell cultures prepared from adult male rats.

Carroll et al. (1991) used rat anterior pituitary cells *in vitro* and determined the apparent half-life of FSHβ mRNA in the presence and absence of recombinant human activin A after addition of actinomycin D.

Demura et al. (1993) measured follistatin-free activin and inhibin in the culture medium of porcine granulosa cells by a competitive protein binding assay and N-fragment RIA, respectively. Both activin and inhibin were secreted under the control of FSH and LH.

Miyamoto et al. (1999) investigated the effect of activin A on secretion of LH, FSH, and prolactin by fe-

male cultured rat pituitary cells at the single-cell level by means of the cell immunoblot assay. Anterior pituitary cells were preincubated with or without activin A for 24 h, after which they were monodispersed and immediately used for cell immunoblot assay.

Peng et al. (1999) examined the expression of activin receptor mRNAs in human ovary and placenta. Primers specific for two type-I and two type-II receptors (ActR-I, Act-RI_B, ActR-II, and Act-RII_B) were used in polymerase chain reaction to amplify cDNAs prepared from granulosa-luteal cells, placental tissues, and trophoblast cells.

Liu et al. (1996) measured release of immunoreactive activin A from cultured rat anterior pituitary cells by a specific radioimmunoassay.

Shintani et al. (1991) developed a radioimmunoassay for the measurement of activin A, which is identical to erythroid differentiation factor.

For measurement of activin in biological fluids by radioimmunoassay, McFarlane et al. (1996) added sodium deoxycholate, Tween 20 and sodium dodecyl sulphate as dissociating agents in order to remove interference by follistatin.

Knight et al. (1996) developed a two-site enzyme immunoassay for the determination of total activin A concentrations in serum and follicular fluid.

Saito et al. (1991) developed an assay method for activin-binding protein, which exploits its high affinity for sulfated polysaccharides, and used this method to investigate the production of activin-binding protein by rat ovarian granulosa cells *in vitro*.

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N.10.8**Follistatin****N.10.8.1****General Considerations**

Follistatin is a monomeric glycosylated polypeptide chain which was identified from bovine and porcine follicular fluids on the basis of its inhibition of pituitary follicle-stimulating hormone secretion (Robertson et al. 1987; Ueno et al. 1987; Ying et al. 1987; Bohnsack et al. 2000). Follistatin exerts its inhibitory effect on FSH secretion by neutralizing activin activity (Namakura et al. 1990; Shimonaka et al. 1991; De Winter et al. 1996). Follistatin is able to bind and neutralize the actions of many members of the transforming growth factor- β family of proteins and plays a significant role during organogenesis (Patel 1998). There are two main forms of mature mammalian follistatin which occur as a result of alternative modes of precursor mRNA splicing, giving core proteins of 315 amino acids and the carboxy-truncated variant of 288 amino acids. Further variants in the molecular weight of mature follistatin occur as a result of varying degrees of glycosylation (Inouye et al. 1991; Sugino et al. 1993).

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N.10.8.2**Immunoassay for Follistatin****PURPOSE AND RATIONALE**

Evans et al. (1998) developed an ultra-sensitive two-site enzyme immunoassay for human follistatin.

PROCEDURE

Female Balb/c mice are immunized subcutaneously with 20 μg recombinant human follistatin rh-FS288 in an emulsion with complete Freund's adjuvant. The immunization is repeated on two further occasions at monthly intervals in complete Freund's adjuvant, before finally boosting intravenously with rh-FS288 (total 100 μg in saline). The spleen is removed and the splenocytes are fused to Sp2/0 myeloma cells using polyethylene glycol following a standard fusion protocol (Galfre and Milstein 1981). Hybridoma supernatants are screened on a 96-well plate coated with rh-FS288 (0.2 $\mu\text{g}/\text{ml}$ in sodium bicarbonate buffer, pH 9.4). Positive clones are expanded and recloned in methyl cellulose. The supernatants from these clones are titrated against rh-FS288 (0.2 $\mu\text{g}/\text{ml}$) under a standard ELISA protocol (Groome et al. 1995). On the basis of these experiments, clones are selected (29/9 and 17/2) and isotoped with a commercial kit. The clones are grown to produce ascitic fluid in pristane-primed BALB/c mice. Purification of IgG is carried out using protein-G affinity chromatography.

Antibody to a selected clone (29/9) diluted in 0.2 M sodium bicarbonate buffer, pH 9.4, is coated by simple absorption onto 96-well ELISA plates overnight at room temperature (10 $\mu\text{g}/\text{ml}$). The following day, the plates are banded to dryness on paper toweling, then 100 μl of dry coat reagent per well is added to the plates. After a 1-h incubation at room temperature the plates are banded to dryness and stored in a sealed box.

Both standard (rh-FS288) and samples are diluted in dissociation solution (84 mmol/l sodium deoxycholate, 3.4% Tween 20, 1% BSA, 5% mouse serum in PBS). Standards are prepared by serially diluting the stock rh-FS288 to give a high standard of 2500 pg/ml and a low standard of 19.53 pg/ml.

Duplicate 50- μl amounts of standard or test samples are added to wells on the plate, which is then sealed

and incubated overnight stationary at room temperature in a sealed moist box. The following day, the plate is washed and to each well is added 50 μ l of approximately 1 μ g/ml of Fab fragment of clone 17/2, which has previously been coupled to alkaline phosphatase by heterobifunctional chemistry (Ishikawa et al. 1983). This is diluted in Tris conjugate buffer: 1% BSA in 25 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.5% Tween 20. After a 2-h incubation in a moist chamber at room temperature, the plate is washed thoroughly, and banded to dryness on paper toweling. Alkaline phosphate substrate (50 μ l) is added to each well and the plate incubated for 2 h stationary at room temperature. Amplifier solution (50 μ l) is then added to each well and the ensuing chromogenic reaction is stopped by adding 0.4 M HCl (50 μ l/well) once color begins to develop in the zero analyte wells and the top standard has an absorbance of approximately 1.8. The well absorbencies are read at 490 nm with a reference wavelength set at 620 nm using a microplate reader.

EVALUATION

To determine whether dose–response relationships of serially diluted standard and test samples are identical (parallel), the slope values (\pm 95% confidence intervals) of log-transformed data for each response curve are compared by linear regression. The curves are deemed to be parallel if the slopes (\pm 95% confidence intervals) are found to overlap.

MODIFICATIONS OF THE METHOD

Nakamura et al. (1992) investigated the effect of follistatin on activin-induced granulosa cell differentiation in freshly harvested granulosa cells from diethylstilbestrol-treated rats. Activin induced a remarkable change in granulosa cellular morphology from elongated fibroblast-like to round cells, which was prevented by follistatin.

Xiao et al. (1992) studied the effects of activin and follistatin on FSH receptors and differentiation of cultured rat granulosa cells *in vitro*.

DePaolo et al. (1991) determined FSH and LH levels by RIA in the serum of ovariectomized rats and compared *in vivo* the FSH-suppressing activity of follistatin and inhibin.

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N.10.9

Further Peptide Hormones Discussed in Chapters Related to the Respective Indications

Further peptide hormones are discussed in chapters related to the respective indications such as:

Chapter A Cardiovascular activity

A.1.1.12.1 Inhibition of Angiotensin-Converting Enzyme *in Vitro*

A.1.1.12.2 Inhibition of Neutral Endopeptidase (Nepriylsin)

A.1.1.14 Angiotensin

A.1.1.15 Renin

A.1.1.17 Endothelin

A.1.1.17.6 Endothelin-Converting Enzyme

A.1.1.26 Adrenomedullin

A.1.1.28 Urotensin

A.1.1.29 Apelin

Chapter E Psychotropic and Neurotropic Activity

E.5.1.16 Neurotensin

Chapter F Drug Effects on Learning and Memory

F.2.0.10 Nerve Growth Factor (Cultured Neurons/Astroglial Cells)

Chapter H Analgesic, Anti-Inflammatory, and Anti-Pyretic Activity

H.1.1.6 Nociceptin

H.1.1.7 Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate

Cyclase-Activating Peptide (PACAP)

- H.2.0.7 Antagonism to Nerve Growth Factor
- H.3.1.2 Bradykinin
- H.3.1.3 Substance P and the Tachykinin Family
- H.3.1.3.3 Neurokinin
- H.3.1.7 Cytokines
- H.3.1.7.3 Interleukin-1 Antagonists
- H.3.1.7.4 Interleukin-1- β Converting Enzyme
- H.3.1.7.5 Nuclear Factor- κ B
- H.3.1.8 TNF-Alpha
- H.3.1.9 Interferon Receptors
- H.3.1.10 Peroxisome Proliferator-Activated Receptors

Chapter J Activity on the gastrointestinal Tract

- J.3.1.6 Gastrin
- J.3.1.8 Gastrin Releasing-Peptide/
Bombesin/Neuromedin
- J.3.1.9 Bombesin
- J.7.0.6 Somatostatin
- J.7.0.8 Secretin
- J.7.0.10 Cholecystokinin

Chapter K Antidiabetic Activity

- K.3 Insulin and Insulin-Like Activity
- K.4.0.1 Glucagon

- K.4.0.3 Glucagon-Like Peptide I
- K.4.0.4 Insulin-Like Growth Factors/Somatomedin
- K.4.0.5 Amylin

Chapter L Anti-Obesity Activity

- L.3.2.2 Uncoupling Protein
- L.4.0.1 Agouti-Related Protein
- L.4.0.1 Melanin Concentrating Hormone (MCH)
- L.4.0.1 Cocaine- and Amphetamine- Regulated Transcript (CART)
- L.4.0.1 Resistin
- L.4.1 Leptin
- L.4.2 Neuropeptide Y
- L.4.3 Orexin
- L.4.4 Galanin
- L.4.5 Adipsin
- L.4.6 Ghrelin

Chapter M Anti-Atherosclerotic Activity

- M.8 Peroxisome Proliferator-Activated Receptors (PPARs) and Liver X Receptors (LXRs)

Chapter 0

Ophthalmologic Activity¹

| | | |
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| O.2.2 | Measurement of Intraocular Pressure by Telemetry | 1920 |
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| O.4 | Experimental Glaucoma | 1922 |
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| O.8 | Safety Pharmacology of Drugs with Ophthalmologic Activity | 1939 |

O.1

Introduction

Ocular pharmacology shares many methods with other indications and treatment of other organs. Therefore, in many instances, the reader has to be referred to other sections, such as:

- neovascularization of the cornea to Sect. A.8.3.4,
- topical anesthesia of the cornea to Sect. G.1.3.1,
- influence on α - and β -adrenoreceptors in the mouse iris to Sect. A.1.3.24,
- *in vitro* determination of carbonic anhydrase inhibition to Sect. C.1.1.1,
- electroretinogram in diabetic hyperglycemia and galactosemia to Sect. K.8.1.5,
- effect of streptomycin induced cataract to Sect. K.8.1.6,
- effect on galactose feeding-induced cataract to Sect. K.8.1.7,
- effect on naphthalene induced cataract to Sect. K.8.1.8,
- immunological methods to Sect. I.2.2.16.

Nevertheless, ocular pharmacology is a special issue, which justifies a special chapter in this book.

O.2

Intraocular Pressure

O.2.1

Acute Measurement of Intraocular Pressure

PURPOSE AND RATIONALE

For evaluation of drugs, intraocular pressure is measured in normal animals or in animals with experimental glaucoma.

PROCEDURE

New Zealand white rabbits, weighing 2.0–2.5 kg, are acclimated to the laboratory environment at least

¹Review and contributions by B. Schultz and W.H. Vogel.

one day prior to experimentation. Intraocular pressure (IOP) measurements consist of topically administering one drop (approximately 50 μ l) of a short-acting local anesthetic (Ophthaine 0.5%) directly on the eyes of an unrestrained rabbit. Approximately 40–45 s following local anesthetic administration, an IOP reading for each eye is taken for 10 s and recorded as mm Hg. IOP is determined by applanation pneumotometry using an Alcon pneumotonometer modified for the rabbit eye (Vareilles et al. 1977).

On the day of the experiment, two baseline IOP measurements are determined and designated as P_1 and P_2 , respectively. The P_1 measurement is recorded 30 min prior to drug administration while the P_2 measurement occurs just prior drug or placebo administration. The P_2 measurement is designated as IOP at zero time and is used as the baseline control value for determination of percent reduction in pressure. Thus, a baseline is determined for each rabbit studied.

Immediately following the P_2 measurement, the appropriate concentration of drug or vehicle is administered topically to the right eye of 5 rabbits. This is done by placing three successive 20 μ l additions directly onto the eye at two- or three-minute intervals. In addition, during drug application, the lower eyelid is held so that excess fluid is collected in the exposed conjunctival sac. Simultaneously, the left eye receives vehicle with the same regimen as the drug-treated right eye. The vehicle (control) treatment is to monitor potential contralateral ocular effects. Following drug or vehicle treatment, IOP measurements are taken at the following time periods: 0.5; 1.0; 1.5; 2.0; 3.0; 4.0; 5.0 and 6.0 h.

EVALUATION

The IOP of each rabbit eye for each time period is initially recorded in mm Hg. However, to account for initial IOP differences among rabbits, the changes in mm Hg are normalized by converting to percent reduction in outflow pressure (ROP). The ROP is calculated according to an equation proposed by Mishima (1981):

$$\% \text{ROP} = (P_o - P_e / P_o - E_p) \times 100$$

where P_o is the IOP reading in mm Hg at zero time, P_e is the experimental IOP reading at the appropriate time following drug or vehicle administration and E_p is the episcleral venous pressure taken as a constant of 10 mm Hg. The percent ROP for each rabbit at each time period is recorded and grouped to

determine a mean percent ROP for each time period as well as the duration of action. The overall activity of the drug based on the mean percent ROP for five rabbits is categorized as follows:

| % reduction in outflow pressure | activity |
|---------------------------------|----------|
| 10.1–20.0 | slight |
| 20.1–40.0 | moderate |
| >40 | marked |

MODIFICATIONS OF THE METHOD

Rowland et al. (1981) studied circadian rhythm in intraocular pressure using repeated tonometry in rabbits.

Santafé et al. (1997) studied the hypotensive effects of topically applied calcium-antagonists on intraocular pressure in conscious rabbits using a manual applanation tonometer calibrated by direct manometry.

Okada et al. (1995) studied the IOP response to different doses of intravitreally injected endothelin-1 in rabbits using a pneumatonometer.

Drago et al. (1997) studied the effect of repeated administration of combinations of beta-blockers with pilocarpine on IOP and heart rate in conscious rabbits.

Chidlow et al. (1999) investigated the effect of topical application of a HT_{1A} receptor agonist on IOP in normotensive rabbits using an applanation tonometer.

Goldblum et al. (2000) studied the effect of an acetylcholinesterase inhibitor on IOP in normotensive rabbits using a TonoPen XL.

The effect of various carbonic anhydrase inhibitors on IOP pressure was measured by several authors, such as Supuran et al. 1999; Briganti et al. (2000).

Burke and Potter (1986) studied the ocular effects of a relatively selective α_2 agonist in cats, rabbits and monkeys. Intraocular pressure was measured using an Alcon pneumatonometer, and pupil diameter under constant illumination using a pupillometer. Furthermore, cat nictitating membrane responses were recorded after injections via the lingual artery into the right external artery supplying the nictitating membrane.

Moore et al. (1995) described long-term non-invasive measurement of IOP in the rat eye. Rats were anesthetized with short-acting isoflurane inhalant and IOP was determined by averaging 15 valid individual readings with a TonoPen 2 tonometer.

Krishna et al. (1995) studied the circadian rhythm of IOP in Lewis rats using a TonoPen 1 tonometer.

Diurnal variations of *IOP* in the eyes of awake male brown Norway rats were measured by Moore et al. (1996) using a TonoPen XL tonometer.

Cabrera et al. (1999) found no significant difference of *IOP* in four strains of rats using conscious, unseeded animals.

Jia et al. (2000) determined the diurnal *IOP* response of Brown Norway rat eyes after sclerosis of the aqueous humor outflow pathways and its relationship to optic nerve damage. Hypertonic saline was injected into a single episcleral vein and *IOP* measured in both the light and dark phases of the circadian cycle in awake animals.

John et al. (1997) developed a method to measure *IOP* in living mice of genetically different mouse strains. Eyes of anesthetized mice were cannulated with a very fine fluid-filled glass micro-needle. The micro-needle was connected to a pressure transducer and the pressure signal was analyzed with a computer system.

Miller and Rhaesa (1996) evaluated the effect of a topical α_2 -agonist on *IOP*, pupil size and heart rate in normal cats.

Bhattacharjee et al. (1999) trained domestic adult cats to accept topical ocular drug administration and pneumotometry. Cats received either various corticosteroids or vehicle topically to both eyes three times a day for approximately 28 days. The increase of *IOP* was found to depend on the concentration and structure of the corticosteroid.

Gelatt et al. (1997) evaluated the effects of locally applied pilocarpine preparations on *IOP* and pupil size in glaucomatous Beagle dogs.

Peterson et al. (1996) compared readings with the Tono-Pen, a handheld applanation tonometer based on the Mackay-Mark principle, with *IOP* measured manometrically after anterior chamber cannulation through the peripheral cornea with a 26-gauge needle connected to a vertically adjustable reservoir and a pressure transducer in cynomolgus monkeys.

For *in vitro* determination of carbonic anhydrase inhibition see C.1.1.1.

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0.2.2**Measurement of Intraocular Pressure by Telemetry****PURPOSE AND RATIONALE**

McLaren et al. (1996), Pericot et al. (1996), Schnell et al. (1996) published methods to measure intraocular pressure by telemetry in conscious, unrestrained rabbits.

PROCEDURE***Telemetric Pressure Transducers***

The implantable pressure monitors (Model TA11PA-C40; Data Sciences International, St. Paul, MN) are cylindrical, measure 25 mm in length and 15 mm in diameter, and weigh approximately 9 g. They contain a pressure transducer, a short-range amplitude modulation radiotransmitter, and a battery in a biocompatible case. Pressure is conducted to the transducer through a fluid-filled polyurethane catheter that is 15 cm long and 0.7 mm in diameter. The 3-mm distal tip of the catheter couples the transducer to the surrounding fluid; it is modified with a thin flexible wall, and the end is filled with a biocompatible gel. The thin wall conducts transient pressure changes, whereas the gel prevents mixing of the fluid in the catheter with the surrounding biological fluid and conducts slow pressure changes.

The transmitter broadcasts an amplitude modulation signal to two antenna and receiver assemblies mounted inside the animal's cage.

Implant Surgery

The transducers are implanted in rabbits weighing 2 to 3.5 kg by using aseptic conditions under anesthesia with intramuscular injections of 50 mg/kg ketamine and 5 mg/kg xylazine. A 4-cm midline incision is made on the dorsal neck and connective tissue and muscle layer over the vertebral column and between the scapulae are bluntly dissected to form a pocket to contain the transducer capsule. This area is covered with moist gauze while the eye is prepared to receive the catheter.

The right eye is used in all experiments. Eyelids are retracted with an infant lid speculum, and a 7-0 silk suture is placed through the mid-corneal stroma approximately 3 mm from the superior limbus. The two ends are weighted to maintain downward rotation of the globe. The conjunctiva is opened 2 to 3 mm temporal to the superior rectus muscle and gently dissected free of the sclera back to the supertemporal orbital rim.

A 10 cm, 17-gauge catheter needle is inserted through the conjunctival opening, advanced to the orbital rim, and pushed through the orbital fascia into the subcutaneous space just behind the orbital rim. It is advanced carefully under the skin to the top of the skull and back between the ears until the tip emerges from the opening at the back of the neck. The needle is flushed with sterile saline to remove any tissue or blood it has collected.

The transducer is dipped in an antibiotic solution (1000 U/ml bacitracin and 10 mg/ml neomycin in Ringer's solution) and is placed in the pocket formed earlier in the neck. The tip of the cannula is then inserted into the bore of the 17-gauge needle and advanced carefully as far as possible. The needle is withdrawn and removed from the conjunctival opening, leaving the transducer catheter along its path. Approximately 2 cm of the catheter is left extending through the conjunctival opening.

The anterior edge of the conjunctival flap is reflected forward, and the sclera over the trabecular meshwork is scraped with a sharp, rounded blade until the trabecular meshwork is visible as a dark streak. A 21-gauge needle is inserted through this thinned tissue into the anterior chamber and withdrawn, and the anterior chamber is refilled through the same opening with Healon. The catheter is grasped with a tube forceps and is inserted through the opening into the anterior chamber. The tip is advanced to approximately mid pupil. Care is taken not to touch the corneal endothelium.

The catheter is secured to the sclera with three 9-0 or 10-0 nylon sutures. Each is tied securely around the catheter three times and its ends passed through the sclera before they are tied together. One 8-0 silk suture is also tied tightly three times around the catheter to promote formation of granulation tissue. The catheter is pulled gently back toward the transducer to remove the excess loop in the orbit.

The capsule is anchored in the pocket on the neck. Again, care is taken, not to nick or make any sharp bends in the catheter. The muscle layer and the skin are closed so that the entire transducer and the catheter assembly are located internally.

For measurement of intraocular pressure lowering drugs, ocular hypertension caused by injection of 5% glucose is used. The rabbits are left undisturbed for 30 min before the beginning of telemetric intraocular pressure measurements. One measurement is recorded every 15 s for 5 s with a sampling rate of 250 Hz. Fifty μ l of test solution or saline are instilled into the conjunctival sac of the implanted eye and intraocu-

lar pressure is recorded for the next 60 min. A sterile 5% glucose solution (20 ml/kg) is then injected into the marginal vein of the ear. Intraocular pressure is recorded for the next 40 min. All studies are performed using a cross-over protocol. A wash-out period of 5 days is allowed between experiments.

EVALUATION

For statistical analysis, the average values of data collected over the time period of interest are calculated for individual animals. For comparisons within groups, statistical analysis is performed on averaged data using two-tailed Student's *t*-test for paired data or unpaired test for unpaired data.

MODIFICATIONS OF THE METHOD

Pericot et al. (1996) induced chronic ocular hypertension by a single injection of α -chymotrypsin into the posterior ocular chamber of rabbits anesthetized by an intramuscular injection of 35 mg/kg ketamine and 4 mg/kg xylazine. Continuous intraocular pressure was measured by telemetry and the effects of timolol, dorzolamide, and epinephrine. The transmitter of a miniaturized radiotelemetry system was implanted into rabbits, and its catheter was tunneled subcutaneously to the superior conjunctival sac and inserted into the mid-vitreous.

Dinslage et al. (1998) recorded the effects of tonometry, handling, water drinking, and instillation of topical ophthalmic medications on *IOP* by telemetry in rabbits for several months during each 24-h day/night cycle.

McLaren et al. (1999) measured the effects of topical ibopramine and epinephrine on the circadian rhythm of *IOP* in undisturbed rabbits by telemetry.

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0.3

Aqueous Humor Flow Rate

PURPOSE AND RATIONALE

Aqueous humor flow rate can be determined by ocular fluorophotometry (Ogdi et al. 1993, 1994).

PROCEDURE

New Zealand White rabbits weighing 2.5–4 kg of either sex are used. For unilateral sympathectomy, the animals are anesthetized with a dose of ketamine/xylazine (44/8 mg/kg i.v.). Through a midline incision in the neck, the cervical sympathetic nerve trunk is isolated from both the vagus and the common carotid artery by blunt dissection. To be sure that the isolated nerve is the sympathetic trunk, it is electrically stimulated with a square-wave pulse of 4 Hz (0.7-ms duration, 5-s train) at 4 V with a bipolar electrode connected to a Grass SD9 stimulator; dilatation of the rabbit's pupil is satisfactory confirmation of nerve isolation. The preganglionic sympathetic trunk and superior cervical ganglion are severed and removed. During 1–2 weeks post surgery, sympathetically denervated eyes of rabbits are examined for persistent miosis and ptosis.

The corneas of adult rabbits are treated bilaterally by instilling 2% fluorescein topically, 1 drop every 5 min for a period of 30 min (Brubaker 1989). Subsequently, fluorescein concentrations in the cornea and anterior chamber are measured using a Fluorotron Master (Coherent Medical Inc., Palo Alto, CA). Basal aqueous humor flow rates are established both in sympathetically denervated and normal eyes. In the following week, groups of rabbits are treated bilaterally with the test drug or with vehicle. Fluorometry recordings are started 30 min after drug or vehicle administration, and subsequent recordings are made every hour for a total of 5 h.

EVALUATION

Student's *t*-tests for paired data are used to evaluate drug-induced changes.

CRITICAL ASSESSMENT OF THE METHOD

The study in hemi-sympathectomized animals allows the separation of direct drug effects from effects exerted via the sympathetic nervous system

MODIFICATIONS OF THE METHOD

According to Yablonski et al. (1978), the acute decrease in intraocular pressure caused by timolol can be explained entirely by its effect on the aqueous humor flow.

The effect of various compounds on aqueous humor inflow into the posterior chamber of the **rabbit** eye can be used to determine the mechanism of action and to compare the potency of *IOP* lowering compounds. Aqueous humor production is indirectly quantified in the New Zealand white rabbit by means of the *IOP* recovery rate assay of Vareilles and Lotti (1981). After a control pneumotonographic *IOP* measurement, a 20% NaCl solution is infused i.v. into the marginal ear vein of a rabbit at a rate of 1 ml/min for 10 min using an infusion pump. The hypertonic saline infusion causes a rapid and marked fall in *IOP* with a peak reduction occurring 15 min after the start of infusion. Thereafter, the pressure gradually returns to normal over the ensuing 120 min in normal rabbits. Compounds under study are solubilized in a suitable vehicle. Sixty min prior to NaCl infusion, the drugs are instilled directly onto both eyes as three successive 20 μ l drops with each drop separated by two or three min. Following the NaCl infusion, the intraocular pressures of both eyes are measured at 5, 10, 15, 30, 60, 90 and 120 min and then every 30 min until *IOP* recovers to preperfusion values. The mean of the *IOP* for the 2 eyes is recorded and plotted against time. The *IOP* recovery rate is then expressed as the slope of the *IOP* recovery line calculated from the points 15 min from the start of NaCl infusion to the ensuing 120 min by means of the least squares method. The difference in slope and percentage change between control and that produced by experimental compounds are analyzed using an unpaired Student's *t*-test. A compound which lowers *IOP* by reducing aqueous humor flow will exhibit a prolonged *IOP* recovery rate as evidenced by a shallower slope compared to that produced by vehicle.

Taarnhøj et al. (1990) reported calibration of measurements *in vivo* of fluorescein in the rabbit cornea. The quenching was measured by four different techniques: (1) by elution of fluorescein, (2) by elution of albumin, (3) by polarization of fluorescence, and (4) by spectrofluorometry. Quenching of fluorescence of fluorescein in the rabbit cornea can be explained by the interaction of fluorescein and albumin in the cornea.

Burke and Schwartz (1996) surveyed the preclinical data on aqueous humor flow and intraocular pressure of brimonidine.

Beilin et al. (2000) characterized the *IOP* lowering activity and possible mechanism of action of a synthetic, non-psychotropic cannabinoid after i.v. administration in rabbits measuring *IOP* by pneumatonometry, aqueous humor inflow rate by fluorophotometry, blood pressure and heart rate by a computerized physiograph system connected to the central ear artery.

Mermoud et al. (1996) established methods to determine normal outflow facility using anterior chamber infusion with constant pressure and aqueous humor production by a technique of dilution with FITC-albumin in Lewis **rats**.

Tian et al. (1997) determined the effects of adenosine agonists on intraocular pressure with a minified Goldmann applanation tonometer and aqueous humor dynamics by fluorophotometry in cynomolgus **monkeys**.

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0.4

Experimental Glaucoma

0.4.1

Alpha-Chymotrypsin Induced Glaucoma in Rabbits

PURPOSE AND RATIONALE

Sears and Sears (1974) induced glaucoma in rabbits by intraocular injection of alpha-chymotrypsin. Several other animal models resembling glaucoma in human beings have been reviewed (Gelatt 1977).

PROCEDURE

Male New Zealand rabbits weighing about 2 kg are pretreated with 10 mg/kg i.p. indomethacin to prevent the otherwise immediate onset of inflammation and

then slightly anesthetized with pentobarbital to eliminate any nystagmus. The right eye is anesthetized topically with 2% lidocaine. The anterior chamber is cannulated with a 30-gauge needle attached to a reservoir set at a pressure of 25 mm Hg. Then a second cannula, 32-gauge, is introduced into the anterior chamber near the limbus and directed to the posterior chamber through the pupil. A sterile isotonic saline solution (0.5 ml) containing 150 units of alpha-chymotrypsin is irrigated through the cannula into the posterior chamber. Care is taken to avoid the injection of any enzyme into the corneal stroma. Both cannulae are then removed without significant loss of aqueous humor. The eyes are examined at daily intervals for the first week, then on alternate days for the second week, and then weekly for the duration of the experiments. Intraocular pressure is measured with a tonometer adapted for rabbit eyes.

Treatment with drugs is performed before and after surgery.

EVALUATION

Intraocular pressure of animals treated with alpha-chymotrypsin and those treated additionally with drugs is expressed as mean \pm SEM and compared statistically with untreated controls using ANOVA followed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Melena et al. (1998a) measured the effect of topical dihydroergocristine on *IOP* in alpha-chymotrypsin-induced ocular hypertensive **rabbits** with a pneumatonometer.

Drago et al. (1997) measured the effects of beta-blockers combined with pilocarpine on rabbit intraocular pressure. Experimental increase of intraocular pressure was induced by a subconjunctival injection of betamethasone-21-phosphate (4 mg/ml) in both eyes every week for 4 weeks. The intraocular pressure was measured in conscious rabbits using an electronic pneumotonometer after surface anesthesia with one drop of 0.4% benoxinate solution.

Melena et al. (1997) induced ocular hypertension in rabbits by weekly subconjunctival injection of a betamethasone suspension into the left eye and measured *IOP* with a manometrically calibrated pneumatonograph.

With the same method, Melena et al. (1998b) measured the effect of topical diltiazem on the rise of *IOP* and the effect on established ocular hypertension.

Santafe et al. (1999) induced ocular hypertension in rabbits by oral administration of 60 ml/kg tap water and measured the effect of topical diltiazem on *IOP*.

Balaban et al. (1997) studied the mechanisms of vasopressin effects on intraocular pressure in anesthetized **rats** after cannulation of the anterior chamber of the eye and infusions of artificial cerebrospinal fluid or arginine vasopressin solutions.

Mermoud et al. (1994) described an animal model of uveitic glaucoma. Uveitis was induced in Lewis rats by injection of S-antigen.

Morrison et al. (1998) produced scarring of the aqueous humor outflow pathways by unilateral episcleral vein injections of hypertonic saline in Brown Norway rats and measured *IOP* and optical nerve damage after topical treatment with artificial tears, 0.5% betaxolol, or 0.5% apraclonidine.

Ueda et al. (1998) described an experimental glaucoma model in the rat induced by laser trabecular photo-coagulation after an intracameral injection of India ink.

Gu et al. (2000) induced experimental glaucoma in the right eyes of Wistar albino rats by intracameral injection of India ink followed by laser trabecular photocoagulation 4 days later. The left eye served as control. Drugs were injected intraperitoneally just before trabecular photocoagulation. Five days later, 3% fast blue was injected into both superior colliculi. The eyes were enucleated another 3 days later and flat mounts of the retinas were prepared. Labeled ganglion cells were counted in the area 1 mm away from the optical disc.

Wang et al. (1997) induced glaucoma in one eye of cynomolgus **monkeys** by repeated argon laser photocoagulation or diode photocoagulation of the mid-trabecular meshwork to study the effects of 5-methylurapidil.

Schmidt et al. (1998) induced glaucoma in rhesus monkeys by 2–3 laser treatments of one eye and measured *IOP*, ocular pulse amplitude and peak pulse blood volume after treatment with anti-glaucoma drugs.

Serle et al. (1998) studied the effect of prostanoid derivatives on *IOP* and aqueous humor flow rates in cynomolgus monkeys with unilateral laser-induced glaucoma.

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- by Woodward and Gil (2004). Shareef et al. (1995), Sawada & Neufeld (1999), Neufeld et al. (1999) described a rat model of chronic elevated intraocular pressure.

PROCEDURE

Male Wistar rats weighing about 250 g are anesthetized with a mixture of 80 mg/kg ketamine and 12 mg/kg xylazine given intraperitoneally. All ocular surgical manipulations are unilateral, the contralateral eye serving as control. A 2-mm-long incision through the conjunctiva and Tenon's capsule is made on the limbal periphery of the eye. Two radial relaxing incisions are made at the edges of the initial incision. The tissue is recessed posteriorly exposing the underlying extraocular muscle, which is isolated and anchored by a suture to expose the underlying episcleral vein. The episcleral veins are identified by their location (slightly deeper than the ocular muscles), relative immobility, larger caliber, branching pattern, the darker blood contained and their communication with a limbal venous plexus. In contrast, the arteries travel a relatively straight course and communicate with a single perilimbal artery. In most cases, four episcleral veins are noted. By manipulating the anchored muscles, a hand-held cautery is applied to the veins. After ocular surgery, the eyes are treated with an antibiotic ointment.

Immediately after surgery, intraocular pressure rises from 12 mm Hg up to at least 20 mm Hg, remaining at this level for months.

This model is used more recently to study biochemical changes during elevated intraocular pressure and/or to study effects of drugs for antiglaucoma effects. Neufeld et al. (1999) treated a group of rats with aminoguanidine, a relatively selective inhibitor of nitric-oxide synthase 2 in the drinking water for 6 months. Tsai (2005) studied the effects of erythropoietin and the preservation of ganglion cells during glaucoma. Sarup (2005) reported on the effects of dorzolamide and timolol on glaucomatous eyes. Guo (2006) studied the effects of glutamate on glaucoma.

EVALUATION

Once a week, each animal is anesthetized (xylazine/ketamine) and intraocular pressure is determined bilaterally using a pneumotonometer. On any given eye, three to five tonometer readings are taken and averaged. On a given day, mean \pm SD is derived for all control and surgical eyes. Significant differences between surgical and control eyes are determined by χ^2 analysis with Student's *t* test for independent means

0.4.2

Episcleral Venous Occlusion Induced Glaucoma in Rats

PURPOSE AND RATIONALE

The importance of trabecular and uvoisceral outflow for the treatment of glaucoma has been underlined

for each day on which measurements are performed. Furthermore, IOP between treated and non-treated animals is compared.

MODIFICATIONS OF THE METHOD

WoldeMussie et al. (2001, 2002) induced chronic ocular hypertension in rats using laser photocoagulation with a blue-green argon laser.

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0.4.3

Photocoagulation Induced Glaucoma in Monkeys

PURPOSE AND RATIONALE

Lee et al. (1985), Woodward et al. (2003) used a method originally described by Gaasterland and Kupfer (1974) for the production of sustained, elevated intraocular pressure in the rhesus monkey by repeated, circumferential argon laser photocoagulation of the mid-trabecular meshwork.

PROCEDURE

Cynomolgus or rhesus monkeys are anesthetized with systemic pentobarbital and placed at the slit lamp delivery system of the argon laser. Both eyes are treated with topical proparacaine 0.5% and photocoagulated using a modified Koeppel-type gonioscopy lens with

hydroxypropylmethylcellulose as the filling fluid. Approximately 200 laser applications are made to each eye, aimed at the middle of the trabecular meshwork, using a 50 μ m beam diameter, 0.2 to 0.5 second duration and 0.4 to 0.8 watts of power. Slit lamp and fundus examination are repeated every 5 to 8 days.

Drugs are applied locally as solutions with a drop size of 50 μ l. Intraocular pressure is recorded in 6-h diurnal curves at 9:30 a.m., 10:00 a.m., 10:30 a.m., 11:30 a.m., 12:30 p.m., 1:30 p.m., 2:30 p.m., and 3:30 p.m. The baseline diurnal curve serves as control and is made 1 or 2 days before the experimental diurnal curve. On the experimental day, baseline intraocular pressure is measured at 9:30 a.m. The test drugs are administered to both eyes of the monkey immediately after the 9:30 a.m. measurement.

This procedure has also been applied by Kielczewski et al. (2005).

EVALUATION

The intraocular pressures on the treated day are compared to that of the control day. Alternatively, the differences in intraocular pressures at intervals after therapy between the treated and control day measurements are compared to the initial (0 hour) differences between the treated and control day values.

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0.5

Local Anesthesia of the Cornea

PURPOSE AND RATIONALE

Surface anesthesia of the cornea is a prerequisite for ophthalmic surgery. Sigmund Freund studied the phar-

macological actions of cocaine and Carl Koller introduced cocaine in 1884 into clinical practice as a topical anesthetic. In pharmacology, following the pioneering work of Sollmann (1918), block of the rabbit corneal reflex as described by Régnier (1923) has become a standard test method for evaluating local anesthetics (Quevauviller 1971; Muschaweck et al. 1986). A detailed description of methods to evaluate the activity of compounds for surface anesthesia is given in Sect. G.1.3.1.

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0.6

Experimental Cataract Formation

PURPOSE AND RATIONALE

Cataract formation can be induced experimentally by various procedures, such as

- **streptomycin** treatment (see Sect. K.8.1.6),
- **galactose feeding** (see Sect. K.8.1.7),
- **naphthalene** treatment (see Sect. K.8.1.8).

Another procedure to induce experimental cataract is treatment of young rats with **selenite**. A review of this model was given by Shearer et al. (1997). Ošťádalová et al. (1978) induced cataract by administration of a single dose of sodium selenite to suckling rats.

PROCEDURE

Male Wistar rats are used. On the 2nd day of life, the sucklings are divided in such a way that 8 males were kept with one mother. On the 10th day of life (i.e., when they are in absolute nutritive dependence of the mother) a single dose of 0.02 M solution of Na₂SeO₃ is administered s.c. to 5 experimental groups receiving 5, 10, 20, 40 and 60 μmoles/kg, respectively. The

first control group receives s.c. a 0.02 M solution of a sulphur compound (Na₂SO₃), i.e., an element homologous with selenium, at a dose of 60 μmoles/kg. As reference group, 2-months-old male rats are used to which a 0.02 M solution of Na₂SeO₃ is administered s.c. in an amount of 20 μmoles/kg. Mothers of the sucklings as well as the group of adult males, are fed on a standard laboratory diet with water ad libitum. All animals are checked daily and the experiment is terminated 20 days after the treatment.

Cataracts in the sucklings are visible by the naked eye after the opening of their eyes, i.e., on the 14–16th day of life. The opacity is yellowish-white, localized in the center of the lens (nuclear cataract). Two forms are observed: permanent cataracts, persisting through the whole experimental period, and intermittent cataracts, which disappear after some time and reappear after a certain delay. Doses of 40 and 20 μmoles/kg are mostly associated with permanent cataract, the dose of 10 μM/kg causes an uneven distribution of both types of cataracts. The cataractogenic effect is not found in adult animals.

EVALUATION

Dose-dependence of the incidence of cataracts is evaluated using the χ^2 -test.

MODIFICATIONS OF THE METHOD

Anderson et al. (1988) observed massive cortical cataract 15–30 days after a single injection of an overdose of sodium selenite into 14-day-old rats.

Watanabe and Shearer (1989) studied the release of crystallins from the lens into aqueous and vitreous humor in rats with a selenite overdose cataract.

Huang et al. (1991) studied DNA damage, repair, and replication in selenite-induced cataract in rat lens.

Huang et al. (1992) observed cataract formation in lenses from rat receiving multiple, low doses of sodium selenite.

Kelley et al. (1993) demonstrated a marked reduction in alpha-crystallin chaperone activity by calpain II in selenite cataract induced in young rats.

Zhao and Shichi (1998) tested the prevention of cataract and other ocular tissue damage induced by injection of 350 mg/kg **acetaminophen** and induction of cytochrome P450 in C57BL/6 mice.

Kojima et al. (1996), Shui et al. (1997) studied cataract formation in Brown Norway rats treated with X-ray irradiation and **corticosteroids** using slit lamp microscopy, photography with a Scheimpflug camera and scanning electron microscopy.

Hatano and Kojima (1996) induced cataract in Brown Norway rats by **UV-B irradiation** and pretreatment with **L-buthionine sulfoximine**.

Ishida et al. (1997) treated adult rats with oral doses of 0.2, 1 or 5 mg/kg **FK506 (tacrolimus)** over 13 weeks. In the highest dose, cataract development was observed.

Cataract formation in **transgenic mice** was studied by Mitton et al. (1996) and by Cammarata et al. (1999).

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0.6.1

N-Methyl-N-Nitrosourea Cataract Induction Sprague-Dawley Rats

PURPOSE AND RATIONALE

Cataracts can be induced in rats by injection of N-methyl-N-nitrosourea (MNU). Yoshizawa et al. (2000) utilized a direct acting alkylating agent to induce cataractous lesions in neonatal stage of critical development. Longstanding evidence of lenticular tissue susceptibility to N-methyl-N-nitrosourea (Murthy et al. 1972, 1979, Roy et al., 1989, and Smith et al. 1988) and retinal tissue susceptibility to N-methyl-N-nitrosourea (Nakajima et al. 1996, Nambu et al. 1997, Taomoto et al. 1998, Yuge et al. 1996) has been documented.

PROCEDURE

Chemical and dose formulation were prepared as follows. N-Methyl-N-nitrosourea (MNU) was purchased from Nacalai Tesque (Kyoto) and kept at -20°C in the dark. Just before use, MNU solution was dissolved in physiological saline containing 0.05% acetic acid. A total of 254 rats (male: 120, female 134) received a single IP injection of 100 mg/kg body weight of MNU at 0 (day of birth), 5, 10, 15, or 20 days of age. For sequential observation the rats treated on days 0 and 5 (groups 1 and 2, respectively) were killed 1, 2, 3, 5, 7, or 14 days after dosing. Those treated on days 10, 15, and 20 (groups 3, 4, and 5, respectively) were euthanized 3, 7, 14, and 30 days after dosing. The rats were observed daily for clinical signs and 8 (group 1) or 4 (groups 2–5) randomly selected rats were weighed on each examination day and euthanized. At each time point, 4 untreated rats were euthanized for comparison.

At necropsy, bilateral eyeballs were macroscopically examined and quickly removed using an operation microscope K-280 (Konan, Tokyo) and complete necropsy was done on all animals.

Acquired tissue was fixed and processed. At each time point 1 eye was fixed overnight in 10% neutral buffered formalin, and the other was fixed either in Davidson solution (2 eyes of each group, in methacarn methanol:chloroform:acetic acid, 6:3:1, 2 or 4 eyes of each group), or in 2.5% glutaraldehyde solution (2 eyes of group 1). The formalin-, Davidson-, and methacarn-fixed tissues were routinely processed for paraffin embedding and stained with hematoxylin and eosin (HE), and selective sections were used for TUNEL staining. Lenses from day 0 MNU-treated rats (group 1) as well as age-matched untreated rats, prefixed in 2.5% glutaraldehyde solution, were postfixed

in 2% OsO₄ and processed for Luveak-812 embedding. One-micrometer thick sections stained with toluidine blue were used to locate areas for electron microscopy. Ultrathin sections stained with uranyl acetate and lead citrate were examined with uranyl acetate and lead citrate were examined with a Hitachi H-600 electron microscope (Hitachi, Tokyo).

EVALUATION

Cataractous lesions are examined by immunohistochemical analysis, in situ detection of apoptosis, DNA fragmentation and Western blotting of Bcl-2 and Bax Proteins, Caspase - 3/ CPP 32 Protease activity assay, and cataract index. Immunohistochemical detection of 7-Methyldeoxyguanosine (7-mdGua) adducts was performed to detect DNA methylamine adducts. Per methods from Sato et al. (1986) eyes from sacrificed rats were fixed in acetone methylbenzoate xylene (Amex) and processed for paraffin embedding. Per methods by Ozaki et al. (1993), deparaffinized sections treated with 0.05 N sodium hydroxide in 40% ethanol for 15 seconds, and 1% nonfat milk in phosphate-buffered saline for 1 hour and then exposed to primary antibody ($\times 1,000$) at 4°C overnight. Immunohistochemistry was carried out using a labeled streptavidin biotin kit (Dako, Carpinteria, CA). 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Wako Pure Chemicals, Osaka) was used for antigen visualization.

In situ detection of apoptosis by TUNEL was carried out using an apoptosis detection kit (Apop-Tag, Oncor, Gaithersburg, MD). Cells with fragmented nuclei in HE-stained sections correlated well with the TUNEL-positive cells. The apoptotic index (number of fragmentation nuclei per whole nuclei $\times 100$) was determined in HE-stained sections and compared among groups. A total of approximately 300 lens epithelial cells located in the germinative zone were counted.

DNA fragmentation was compared with apoptotic methods. The genomic DNA was isolated from the excised lenses (each sample contained 8 whole rat lens with the use of a SepaGene kit (Sanko, Tokyo) based on the guanidine thiocyanate extraction procedure. Per methods from Nambu et al. (1998) an equal amount of DNA sample (10 $\mu\text{g}/\text{lane}$) was subjected to electrophoresis on a horizontal 0.8% agarose gel containing ethidium bromide and visualized under ultraviolet illuminations.

Western Blotting of Bcl-2 and Bax proteins was performed to detect downregulation and upregulation, respectively as a downstream expression within the apoptotic cascade. Lenses were minced and homog-

enized in PBS (-). The homogenate was sonicated 3 times and centrifuged at 14,000 $\times g$ for 30 minutes. Per methods by Laemmli (1970) the supernatant was diluted with lysis buffer, and tissue extracts containing 50 μg of total protein were subjected to 12% SDS-PAGE. Proteins were blotted to nitrocellulose membrane (Bio-Rad, Hercules, CA). After the nonspecific protein binding had been blocked with 5% nonfat dry milk in PBS for 30 minutes, blots were incubated at 4°C overnight in antimouse/rat Bax (PharMingen, CA, $\times 200$ dilution in PBS with 1% BSA) or anti-Bcl-2 (PharMingen, CA, $\times 200$) antibody. After being washed 3 times in buffer containing 1% Triton $\times 100$, blots were incubated for 30 minutes in EnVision + ($\times 20$, Dako Glostrup) and treated with DAB for color development. The density of each band was evaluated by computer-associated scanner densitometry (NIH image).

Caspase-3/ CPP 32 Protease activity assay was used to examine for CPP-32 activation after lens epithelial cells and to differentiate a-nucleated lens fibers (Ishizaki et al. 1998). Caspase 3 activity of lenses was assayed using caspase 3/ CPP32 colorimetric protease assay kit (MBL, Nagoya). Same protein samples used for western blotting were homogenized in 500 μl of lysis buffer, and homogenates were centrifuged at 10,000 $\times g$ for 1 minute. Supernatants were prepared to 100 μg protein/50 μl lysis buffer, and 50 μl were added to reaction buffer and incubated for 2 hours at 37°C with 200 μM DEVD (Asp-Glu-Val-Asp)-p NA substrate (MBL, Nagoya). Reactions were monitored in a microplate reader at an excitation wave length of 405 nm.

Cataract index was assigned to determine severity of cataract. In routinely prepared HE-stained tissue sections, bilateral eye specimens were evaluated based on the presence of the following 7 items: 1) lens epithelial apoptosis, 2) lens epithelial desquamation 3) multilayered spindle epithelium, 4) lens fiber swelling, 5) liquefaction/vacuolar change, 6) calcification and 7) bizarre nuclei of lens fiber cells. Each item was arbitrarily scored as absent (point 0), slight (point 1), moderate (point 2), severe (point 3), and very severe (point 4). The cataract index was the sum of each score.

MODIFICATIONS OF THE METHOD

Kiuchi et al. (2002) altered the age at injection and confirmed presence of cataracts by histological methods. Cataract was induced by single intraperitoneal injection of N-methyl-N-nitrosourea in 15-day-old Sprague-Dawley (Jcl:SD) rats. Threshold dose

of MNU required for cataract induction in a 3–4 week time period was 70 mg/kg. Males and females were both equally affected. Mature cataract was confirmed histologically by degeneration, swelling, vacuolation, liquefaction of the lens fibers and formation of Morgagni-like water vacuoles.

Miki et al. (2006) used the model to compare the effects of differences in gender and gonadal status on the occurrence of N-methyl-N-nitrosourea induced cataract and retinopathy in Lewis rats. Ovary-intact, ovariectomized, testis-intact and testectomized rats were used for the study. Castration was performed at 36 days of age, 50 mg/kg MNU was administered intraperitoneally at 50 days of age and lens and retinal changes were evaluated at 260 days of age (210 days after MNU injection). Results were assessed by cataract index and retinal damage ratio.

Kiuchi et al. (2003) adapted cataract model to examine retinal degeneration following single injection of 60 mg/kg body weight of N-methyl-N-nitrosourea into Sprague-Dawley rats. Retinal degeneration was confirmed by electroretinogram (ERG) recordings and morphometric analysis of retinal thickness and damaged retinal area. For ERG, animals were dark-adapted for 12 hours and prepared under dim red light. ERGs were recorded with a gold wire loop on the cornea using 1% tetracaine topical anesthesia and a drop of 1% methylcellulose. A gold wire electrode was placed on the sclera 1 mm from the temporal limbus. Ground electrode was placed on the ear. Signals were band-pass amplified using a computer-assisted signal averaging system. Scotopic ERG responses averaged with a stimulus interval of 3 to 60 seconds and 20–30 scotopic responses were averaged with a stimulus interval of 1 second. Strobe flash stimuli were presented in a Ganzfeld bowl (Model GS2000; LACE Electronica sel via marmicilo, Pisa, Italy). Immediately after ERG animals were sacrificed and morphometric analysis was completed. Tissues were fixed and processed as above. Then, color images of stained sections were obtained as PICT files with a Fujix HC-2000 high-resolution digital camera (Fujifilm, Tokyo, Japan). Brightness and contrast of each image file were uniformly enhanced using Adobe Photoshop version 4.0 and images were analyzed using NIH imaging free-ware. Total retinal thickness (from the internal limiting membrane to the pigment epithelium) and photoreceptor cell thickness (thickness of the outer nuclear layer and photoreceptor layer were measured and the photoreceptor ratio (photoreceptor cell thickness/total retinal thickness \times 100) was calculated as described by Yoshizawa et al. (2000). Measurements were taken at

the posterior pole (approximately 200 μ m on either side of the optic nerve). Area of retinal damage was determined from sections prepared at megaloscopic magnification and color images obtained as PICT files using high-resolution digital slide scanner (Dimage Scan Multi; Minolta, Tokyo, Japan). Entire length of retinal and length of damaged area were measured on brightness and contrast enhanced images. Damaged area was defined as the presence of fewer than 4 photoreceptor cells in the outer nuclear layer and the retinal damage ratio was defined as length of damaged retina/length of whole retina \times 100.

CRITICAL ASSESSMENT OF THE METHOD

Most animal models cause more or less complete opacification whereas human cataracts start as many focal points in different lens layers. Human lens layers include: anterior capsule, cortex, nucleus, and posterior capsule. Age related human lenticular cataractous changes are localizable in these layers and termed capsular opacities, anterior cortical cataracts, nuclear sclerosis and posterior subcapsular cataracts respectively.

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0.6.2

TGF β Cataract Induction in Wistar Rats

PURPOSE AND RATIONALE

Hales et al. (1999) used TGF β normally secreted by transforming cells to stimulate growth of normal cells for cataract induction. Previous studies by Lui et al. (1994) and Hales et al. (1994) reported that TGF β specifically stimulates growth of lens epithelial cells evidenced by aberrant morphologic and molecular changes that mimic those documented in some forms of human cataract.

PROCEDURE

Male Wistar rats were killed at 15 weeks, one at 6 weeks and one at 12 weeks. Per methods by Schulz et al. (1996), following euthanizing, eyes were removed and placed in culture dishes containing Medium 199 (Trace Biosciences, Sydney, Australia) with BSA and antibiotics.

EVALUATION

Both eyes from four rats were fixed whole for routine histology to determine the influence of TGF β

on all ocular tissues. Lenses removed from remaining eyes as whole lens cultures then examined for the presence of opacities and photographed. Representative lenses and whole eyes were fixed in Carnoy's (1:3 acetic acid: ethanol) and processed for routine histology and immunolocalization. Briefly, entire lenses and whole eyes were serially sectioned and collected onto subbed glass slides. Every fifth slide was stained with hematoxylin-eosin (HE) and sections were examined for evidence of plaque formation and/or fiber deterioration. Sections corresponding to those that displayed histological changes were used for immunolocalization of β -crystallin and the extra cellular matrix proteins laminin and type I collagen. Nuclei were counterstained (Hoechst 33258; Boehringer-Mannheim, La Jolla, CA).

Photographed lenses were examined for displaying loss of clarity and changes in cellular architecture. Cell rupture of fibers if evident would be a result of needle tip penetration of capsule rather than an alteration induced by TGF β .

Micrographs of lenses were assessed for transparency. Lack of transparency was detectable as the presence of distinct subcapsular opacities toward the anterior and/or posterior poles of the lens, or a generalized more diffuse clouding involving the cortical fiber cells.

Histological examination of serial sections compared controls vs. TGF β eyes. Control eyes maintained a normal lens equator and bow zone and eyes injected with TGF β revealed the characteristic appearance of lens equator and bow zone disruption histologically. An abnormal finding in eyes injected with TGF β may also be evidenced by nucleated fiber cells in the lens cortex and extending toward the posterior pole. Mikuni (1980) and Vrensen (1995) have recognized that severe disruption of the ordered arrangement of nuclei at the rat lens equator and in the bow region as well as rat lenses that atypically retain their nuclei beyond the bow region resemble the changes in lenses associated with human cortical cataract. Finally, cataractous growth from TGF β injected eyes was viewed for their tendency to lose their uniform curvature, as depicted by an undulating capsule.

HE stained sections were observed for plaque formation. Positive plaque formation is demonstrated in slides containing subcapsular plaques with cells appearing rounded and containing large, dense nuclei or others present with matrix deposits where cells appear flattened and slightly elongated except for a layer of epithelial-like cells beneath the matrix deposits.

Immunolocalization following previous methods established by Hales et al. (1995) was performed on HE stained sections that were histologically positive for abnormalities. Sections were assessed immunologically for deposits of laminin and type I collagen by reactivity with laminin.

MODIFICATIONS OF THE METHOD

Fujinaga (1990) conducted long-term observations on 180 Wistar male rats to demonstrate similarities between the senile Wistar rat cataract and the human senile cataract. Other variables under study included agents thought to slow the progression of cataracts. Rats were initially classified into six groups (control, vitamin E diet, ARI, EPC eye drops, catalin eye drops and reduced catalin eye drops) to study the effects of the agents on the cataract. Effects of anti-cataract agents were investigated using cultured lens epithelial cells.

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employed the diabetic cataract model to study apoptosis in lens epithelial cells.

PROCEDURE

Sprague-Dawley rats that were three weeks old were intraperitoneally injected with 0.1 mg/g bodyweight of streptozotocin (Sigma, St. Louis, MO, USA) to prepare a rat diabetes model. All rats weighed 50 g. Every other week between 4 and 12 weeks of age the lens anterior capsule was excised for subsequent epithelial proliferation studies. A total of 60 rats were used; twelve rats were used each time. Diabetic inclusion criteria were defined as a blood glucose level of 200 mg/dL or higher, which was measured twice: One week after streptozotocin administration and prior to excision of the anterior capsules. Rats were sacrificed with ether anesthesia and the eyeballs were immediately extracted, incised at the posterior lens capsular side, and the anterior capsules with lens epithelial cells were obtained.

Once the rat diabetes model was established, immunostaining of proliferating cell nuclear antigen (PCNA) was performed. This is an index of nuclear DNA synthesis. Each sample was fixed in 0.1M phosphate buffer (pH 7.4) containing methanol for anti-PCNA immunohistochemical analysis.

To perform immunohistochemical staining of PCNA the avidin-biotin complex method (ABC method) was applied. ABC method utilizes a Histofine SAB-PO(M) kit (Nichirei, Tokyo) and anti-PCNA monoclonal antibody (mouse monoclonal antibody NC012; Novocastra Lab, Newcastle, UK) as the primary antibody. Samples without treatment with anti-PCNA monoclonal antibody represented the negative control in each staining. Light microscopy via a differential interference prism (Olympus, Tokyo) was completed to observe fragments.

EVALUATION

Diabetic cataracts of the anterior lens capsules are examined by immunohistochemical analysis. Sprague-Dawley rats injected with streptozotocin can serve as a model for the formation of diabetic cataracts and studying lens epithelial apoptosis. In the absence of high glucose levels the non-diabetic lens epithelial cells survive independently of signals from other cells and produce a factor that inhibits cell death or apoptosis (Ishizaki et al. (1993), Raff et al. (1993).

MODIFICATIONS OF THE METHOD

Gavrieli et al. (1992) observed lens epithelial proliferation and detected an amount of fragmented DNA rep-

0.6.3

Diabetic Cataract Formation in Sprague Dawley Rats

PURPOSE AND RATIONALE

Diabetic cataract formation was induced in rats by injection of streptozocin. Takamura et al. (2001) em-

resenting apoptotic cell bodies by using the TUNEL method. In this method, diabetic cataracts are induced as above and the anterior capsule is excised as above. Then each sample was fixed in 0.1M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for the Terminal deoxynucleotidyl transferase (TdT-mediated dUTP-biotin nick end labeling (TUNEL) method for immunohistochemical analysis. Specimens of anterior capsule were then extended on glass slides and treated with 10-fold diluted proteinase K (DAKO, Glostrup, Denmark). ApopDETEK (DAKO) was used for TdT reaction, and Horseradish Peroxidase-DAB in situ Detection System (DAKO) was used for reaction of biotin-labeled dUTP with peroxidase-avidin complex. Samples without treatment with TdT represented the negative control in each staining.

Umemura et al. (1996) extended the duration of sample fixation and concentration of proteolytic enzymes. This may increase the ratio of TUNEL-positive cells to whole fragments which would improve the relationship between the types of cataract and diabetes. The results show that DNA is fragmented over time in fixed preparations so that an increased duration may be necessary.

Hegde et al. (2003) investigated the suitability of using CD-1 mice, a species known to have low lens aldose reductase activity as a streptozotocin diabetic cataract induced model. The mice were injected with streptozotocin and blood glucose evaluated seven days post injection. Diabetic inclusion criteria were defined as a blood glucose level of 300–400 mg/dL or higher. Animals were examined by direct ophthalmoscopy, slit lamp examination and Scheimpflug photography. Additionally morphological studies were performed to view multilayered and numerous nucleated cells in the superficial anterior cortex.

Lee et al. (1994, 1999) compared diabetic cataract formation in transgenic mice that overexpress aldose reductase. The entire 1.4-kb cDNA from a human AR (hAR) clone with 36-bp 5' and 325-bp 3' untranslated regions (16) was released from the Bluescript vector by Xba I and EcoRV digests and inserted into the Xba I and Msc I site of the pCAT-Basic vector (Promega) that contains the simian virus 40 splice site and poly (A) sequence. A single intraperitoneal injection of streptozotocin at a dose of 200 mg/kg of body weight induced hyperglycemia. Lenses were examined through a dilated pupil. The study showed that these transgenic mice that overexpress aldose reductase in their lens epithelial cells were found to become susceptible to the development of diabetic and galactose cataracts.

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0.6.4

Cataract Formation in Knock Out Mice

PURPOSE AND RATIONALE

Cataract formation was studied in genetically altered mice to detect factors influencing cataract origination and progression. Brady et al. (1997) studied the influence of α A-crystallin (α A), a dominant protein in the lens, on cataract initiation and development.

PROCEDURE

α A Gene knockout mice were generated as follows. A λ phage clone spanning 16 kb of the α A locus was isolated from a 129Sv mouse genomic library (Stratagene). A targeting vector was constructed by placing the following pieces of DNA, in the given order, into the plasmid pBluescript (Pro-mega): a 9-kb NotI/AatII restriction fragment encompassing the 5' flanking region and first nine nucleotides of the α A coding region, the phosphoglycerate kinase neomycin phosphotransferase (PGK/neo) expression cassette from the vector pPNT (24) in opposite transcriptional orientation to the α A gene, a 1.3-kb XhoI/XmnI restriction fragment containing most of the first intron and part of the second exon of the α A gene in the same orientation as the α A promoter fragment, and the PGK/herpes simplex virus thymidine kinase expression cassette of pPNT in the same orientation as the α A sequences. In addition, to ensure that no protein-encoding transcripts

could be produced by the disrupted allele, a double-stranded oligonucleotide containing translation termination codons in all three reading frames as well as a copy of the αA polyadenylation signal was inserted between the 5' αA sequence and the PGK/neo cassette. Linearized knockout plasmid DNA (30 mg) was electroporated into 4×10^7 J1 embryonic stem cells derived from 129Sv mice. Cell lines established from 192 colonies surviving double selection with the neomycin analogue G418 and ganciclovir were analyzed by PCR for homologous recombination at the αA locus. Correctly targeted embryonic stem cells, confirmed by Southern blot analysis, were injected into C57BL/6 blastocysts and then implanted into pseudopregnant CD1 female mice. Chimeric males were mated to 129Sv or B6D2F1 females, and heterozygous offspring identified by PCR were interbred to produce mice homozygous for the αA knockout allele.

Mice that were homozygous for the disrupted allele produced no detectable αA in their lenses. Initially, the αA -deficient lenses appeared structurally normal, but they were smaller than the lenses of wild-type littermates. Later on, the lenses developed an opacification that started in the nucleus and progressed to a general opacification with age.

EVALUATION

Eyes were examined by slit lamp, histo- and immunohistochemistry and protein analyses. It is suggested that such knock-out mice can serve as a model for studying the formation and drug treatment of cataracts.

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0.7

Models of Eye Inflammation

0.7.1

Allergic Conjunctivitis

PURPOSE AND RATIONALE

Allergic conjunctivitis is a common clinical condition that resembles immediate hypersensitivity reactions in the nose, airways, and skin in many aspects. Allergic conjunctivitis is regarded as a mast cell-mediated disease: a similar array of inflammatory mediators, mostly histamine, is released, and the conjunctiva is responsive to many of these inflammatory mediators.

Trocme et al. (1986), Fukushima et al. (1997), Iwamoto et al. (1999) induced allergic blepharokeratoconjunctivitis in rats by immunization with ovalbumin. Keane-Myers et al. (1999) sensitized and challenged mice by local application of a cat dander extract. Ragweed pollen was used for sensitization of mice by Merayo-Llives et al. (1996), Hu et al. (1998), Magone et al. (1998, 2000).

PROCEDURE

An extract of ragweed pollen is prepared. Ragweed is defatted with acetone and extracted with 50 mM Tris buffer overnight. The extract is then dialyzed against PBS for 48 h, filtered, and stored at 4°C until use. Protein concentration is determined with Coomassie plus protein assay. The ragweed extract is biotinylated. An Al(OH)₃ gel (alum) is prepared, centrifuged for 15 min at 1000 rpm and stored at 4°C. Ragweed and alum are mixed at a concentration of 50 μ g ragweed in 5 mg of alum 40 min before immunization. On day 0 mice are anesthetized with methoxyflurane during the injection of ragweed and alum in one hind footpad. On day 10, conjunctivitis is induced by topical application of 1.5 mg ragweed suspended in 10 ml phosphate buffered saline, pH 7.2, into each eye. Two groups of control mice are either not immunized or immunized with alum alone; both groups are challenged with the same dose of ragweed on day 10. The animals are sacrificed 20 min and 3, 6, 12, 24, 48, and 72 h after topical ragweed administration.

Animals are examined clinically for signs of immediate hypersensitivity response 20 min after the topical application of ragweed. Chemosis, conjunctival redness, lid edema, and tearing were each graded on a 0–3+ scale. The total score consists of the sum of scores in each of these four categories.

For histology, the eyes are enucleated with the attached lids and intact conjunctiva and immediately fixed in 4% glutaraldehyde for 30 min and then transferred to 4% formaldehyde for at least 24 h before processing. The tissue is embedded in methyl acrylate, serially sectioned and stained. The numbers of infiltrating neutrophils, eosinophils, goblet cells, and mast cells in one section are counted. Immunohistochemical staining is performed with rat primary antibodies using an avidin-biotin-peroxidase complex technique. Macrophages and lymphocytes in one section of the conjunctiva are assessed.

EVALUATION

All results are presented as mean \pm SE. Comparisons between immunized mice, drug treated mice

and controls are made with the two-sided Student's *t*-test.

MODIFICATIONS OF THE METHOD

Hu S et al. (1998) compared effectiveness and molecular pharmacological mechanisms of antiallergic agents on experimental conjunctivitis induced by ragweed in SWR/J mice. Allergic conjunctivitis was evaluated by scoring of the clinical signs and histopathology. mRNA expression of interleukin 1β , IL-6 and tumor necrosis factor α was analyzed by reverse transcription polymerase chain reaction techniques.

Woodward et al. (1995) studied the pruritogenic and inflammatory effects of prostanoids in the conjunctiva in **guinea pigs** and counted the scratching episodes by the hind limb.

Reichel et al. (1998) established a simple model of **conjunctival wound healing** in the mouse eye. Four week old BABL/c mouse eyes were studied over a 14 day period. Surgical procedure under general anesthesia involved a blunt dissection of the conjunctiva performed by injection of 25 μ l of phosphate buffered saline via a 27 gauge needle into one eye, while the other was used as control.

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0.7.2

Corneal Inflammation

PURPOSE AND RATIONALE

Unlike the conjunctiva, the cornea is an avascular tissue. The cornea serves three major functions: protection, refraction and light transmission. Corneal inflammatory diseases usually result from injury and infection rather than from allergic disorders. Models involving chemical or mechanical injury, therefore, directly mimic clinically encountered situations.

Leibowitz and Kupferman (1974) induced inflammatory response by the injection of clove oil directly into the corneal stroma, resulting in the chemotactic attraction of large numbers of polymorphonuclear leukocytes.

PROCEDURE

New Zealand albino rabbits of either sex are anesthetized with 15 mg/kg i.v. sodium thiamylal. A corneal inflammatory response is produced in both eyes by the interlamellar inoculation 0.03 ml of laboratory grade clove oil centrally with a 30-gauge needle attached to a tuberculin syringe. All animals receive two intravenous injections of 0.05 mCi/kg of tritiated thymidine (0.7 Ci/mM) 24 h apart. The second thymidine injection is given 24 h prior to the induction of the corneal inflammatory response. Therapy is initiated immediately after the intracorneal injection of clove oil consisting of one drop (0.05 ml) of test compound or standard every hour for a total of 6 doses. One h later, the animals are sacrificed under anesthesia and a 10 mm penetrating corneal button is removed by

trephination. The tissue samples are solubilized with a commercially available solubilizing agent (Soluene, Packard Instruments Corp.). The samples are counted in a scintillation counter for 10 min and the amount of radioactivity in each cornea documented.

EVALUATION

The data from individually treated eyes are expressed as percent change in radioactivity relative to their own untreated control eyes. These differences are averaged to determine a mean value for each treatment group. Statistical comparisons are performed by standard methods including random block analysis.

MODIFICATIONS OF THE METHOD

Neovascularization of the rabbit cornea has been used by several authors to study inhibition of angiogenesis (see Sect. A.8.3.4).

Alio et al. (1994, 1995) studied the relative effectiveness of nonsteroidal anti-inflammatory drugs versus corticosteroids and of antioxidants in the topical treatment of experimental acute corneal inflammation induced by corneal alkali burn in rabbits. The corneal inflammatory response was evaluated by Luminol-enhanced chemiluminescence, ultrasonic pachymetry, and computer-assisted analysis of the area of corneal ulceration.

Laria et al. (1997) evaluated combined non-steroidal therapy in experimental corneal injury. A corneal alkali burn was induced in New Zealand white rabbits by applying 1-N NaOH filter paper on the central axis of the right cornea for 30 s. The inflammatory index, area and perimeter of the wounded corneal zone, and corneal transparency were evaluated after 5 days treatment.

A similar method was used by Ozturk et al. (2000).

Sonoda et al. (1998) studied inhibition of corneal inflammation by the topical use of Ras farnesyltransferase inhibitors. The central corneas of BALB/c mice were cauterized with silver nitrate. Clinical signs and neovascularization were evaluated.

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0.7.3

Auto-Immune Uveitis in Rats

PURPOSE AND RATIONALE

The mechanisms of certain types of uveitis have been studied in animals involving autoimmunity. Uveitis has been produced in guinea pigs following injections with homologous uveal tissue.

PROCEDURE

Hartley strain guinea pigs, weighing 400–500 g, are injected at 0, 1, 2, 5, and 8 weeks with a 10% suspension of homologous retina emulsified in Freund's complete adjuvant. The retinas are obtained by dissection of freshly removed eyes of either normal albino or pigmented guinea pigs and stored frozen at -50°C . Each animal receives 0.2 ml of this emulsion at a time, the first injection being administered intradermally into both hind foot-paws, and the remainder of the series given intramuscularly. Groups of 6 animals receive the test drug or the vehicle orally. Control animals receive similar injections of saline. The severity of the uveal inflammation can be clinically evaluated by slit lamp examination. The animals are sacrificed and the eyes are examined histologically.

EVALUATION

The incidence of uveitis is recorded by histological means as percentage of treated animals.

MODIFICATIONS OF THE METHOD

Highly purified bovine retinal S antigen in complete Freund's adjuvant was injected subcutaneously in **guinea pigs** to induce experimental autoimmune uveitis (Liversidge et al. 1987; Mahlberg et al. 1987).

Cultured retinal pigment epithelial cells from guinea pigs were used by Liversidge et al. (1988).

Experimental autoimmune uveitis has been induced in **rats** (Nussenblatt et al. 1981; Chan et al. 1984, 1987; Mochizuki et al. 1985; Fujino et al. 1988).

Experimental autoimmune uveoretinitis was in-

duced in Lewis rats by injection of very high doses of bovine opsin (Brockhuysse et al. 1987).

Melanin-associated antigen from the iris and ciliary body was used to induce experimental autoimmune anterior uveitis in male Lewis rats by Bora et al. (1995).

Ramanathan et al. (1996) found that recombinant IL-4 aggravates experimental autoimmune uveoretinitis in Lewis rats.

Hanashiro et al. (1997) injected various doses of either lipopolysaccharide or the lipid A region of the lipopolysaccharide into the foot pad of an inbred strain of Lewis rats and compared the inflammation patterns by assessing the protein concentrations and the inflammatory cell content in the aqueous humor.

Merino et al. (1998) reported that lipoteichoic acid from *Staphylococcus aureus* injected into the foot pad of Lewis rats induced a strong ocular inflammation between 24 and 30 h after injection.

Hikita et al. (1995) evaluated the effects of topical FK506 on endotoxin-induced uveitis in the Lewis rat.

Tsuji et al. (1997) investigated the effects of systemically and topically applied betamethasone derivatives on endotoxin-induced uveitis in female Lewis rats.

McMenamin and Crewe (1995) studied kinetics and phenotype of the inflammatory cell infiltrate and the response of the resident tissue macrophages and dendritic cells in the iris and ciliary body in endotoxin-induced uveitis in Lewis rats.

Uchio et al. (1997) achieved suppression of actively induced experimental autoimmune uveoretinitis in rats by CD4⁺ T cells.

Egwuagu et al. (1999) found in transgenic Lewis rats that expression of interferon-gamma in the lens exacerbates anterior uveitis and induces degenerative changes in the retina.

Immunogenic uveitis in **rabbits** was studied by Kaswan and Kaplan (1988).

Jamieson et al. (1989) sensitized rabbits to bovine serum proteins and then challenged intravitreally to induce an uveitis. They recommended this procedure as a model for anti-inflammatory drug screening.

Whitcup et al. (1996) produced endotoxin-induced uveitis in **mice** by injecting 200 µg *Salmonella typhimurium* endotoxin diluted in 0.1 ml sterile saline into one hindpad.

Geiger et al. (1994) reported that transgenic mice expressing IFN-gamma in the retina develop inflammation in the eye and photoreceptor loss.

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0.7.4

Ocular Inflammation Induced by Paracentesis

PURPOSE AND RATIONALE

Paracentesis stimulates prostaglandin PGE₂ and PGF_{2α} release into the anterior chamber of the eye which can be inhibited by antiinflammatory agents (Miller et al. 1973; Kulkarni and Srinivasan 1985).

PROCEDURE

New Zealand white rabbits (2.0 to 2.5 kg) are anesthetized with a 50:50 mixture of xylazine (20 mg/kg) and ketamine (100 mg/kg) i.m. Test compounds are topically administered by placing three drops (about 60 μl) on each eye. The eyes are then taped shut to prevent drying. One hour after compound or vehicle administration, inflammation is induced by paracentesis. For each rabbit, the aqueous humor from both eyes is combined to form the “Pre” sample. Three more

drops of compound or vehicle are again administered to each eye and the eyes taped shut. Another one-hour period is allowed to elapse, enough time for the aqueous humor to build back up in the eye. Paracentesis is performed a second time and the aqueous humor from both eyes is combined to form the “Post” sample. Prior to this second paracentesis, the general appearance of the eye is rated on a scale of 1 to 3, based on the following criteria: 1 = clear, relatively normal appearance; 2 = opaque, some protein accumulation in the anterior chamber; 3 = very cloudy, protein accumulates in clumps in the anterior chamber, hyperemia, swelling. This rating gives some indication of the extent of inflammation and correlates with the amount of prostaglandin accumulation. Prostaglandins are measured in 10 μl of aqueous humor from the “Pre” and “Post” samples by RIA.

EVALUATION

The amount of prostaglandin present is determined according to the standard curve supplied with each RIA kit. The extent of inflammation is determined by the magnitude of increase in prostaglandin levels in the “Post” versus the “Pre” sample. Dose-response curves for inhibition by test drug and standard are established.

MODIFICATIONS OF THE METHOD

Struck et al. (1995) studied the effects of non-steroidal anti-inflammatory drugs on inflammatory reactions. The right eye of rabbits was burned with alkali (0.25 M sodium hydroxide). The animal were treated with eye drops containing a cyclooxygenase inhibitor and a leukotriene antagonist. Hyperemia of the limbal vessels, corneal vascularization, the number of PMNLs in the cornea and the prostacyclin level in the anterior chamber of the eye served as criteria.

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0.7.5**Ocular Inflammation by Lens Proteins****PURPOSE AND RATIONALE**

Ocular inflammation can be induced by lens proteins which can be prevented by lipoxygenase inhibitors (Miyano and Chiou 1984; Chiou and Chiou 1985; Chang and Chiou 1989).

PROCEDURE

New Zealand albino rabbits are anesthetized by i.m. injection of 25 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. The anesthesia is maintained by injection of 12.25 mg/kg ketamine and 2.5 mg/kg xylazine every hour throughout the experiment. After the animals are deeply anesthetized, 50 μ l of the drug is topically applied to one eye and the other eye receives 50 μ l of the solvent. One hour after the application of the drug, 25 μ l of lens protein (30 mg/ml, prepared according to Miyano and Chiou 1984) is injected intracamerally with a 27 1/2 gauge needle; extreme care is taken to avoid contact with the iris. Fifteen min after injection of lens proteins, 14 mg/kg of fluorescein is infused through the marginal ear vein at a rate of 1 ml/min. The inflammation which occurs in the eyes of the rabbits is measured by quantifying the amount of fluorescein leakage into the anterior chamber using a fluorophotometer. Measurements are taken at various intervals after injection of lens proteins. At the end of experiments, the animals are sacrificed by an overdose of pentobarbital sodium. Fluorescence measurements are done at 30, 60, 90, 120, 180, 240 and 300 min intervals.

EVALUATION

The concentration of fluorescein in the anterior chamber is determined by taking the average of three values in each of the fluorometric measurements. The results are analyzed by using a paired *t*-test with *P*-values of less than 0.05 as statistically significant.

MODIFICATIONS OF THE METHOD

Augustin et al. (1996) evaluated the effects of allopurinol in lens induced uveitis by morphological methods and compared these effects with those of steroids and a combination of both drugs biochemically and morphologically.

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0.7.6**Proliferative Vitreoretinopathy in Rabbits****PURPOSE AND RATIONALE**

Injection of cultured fibroblast cells into the rabbit vitreous results in a condition that mimics the features of proliferative vitreoretinopathy in man (Alvarez and Kock 1976; Sugita et al. 1980; Ophir 1982). Proliferative vitreoretinopathy is a complication of severe eye injury or retinal detachment and is a major cause of failure in retinal reattachment surgery. Because of its simplicity and reproducibility, Wiedemann et al. (1984) proposed this model in rabbits as screening model for antiproliferative drugs.

PROCEDURE

Dermal fibroblasts are obtained from skin explants of young rabbits which are prepared by excising a 1 \times 1 cm area of dorsal skin and scraping it thoroughly with a scalpel blade to remove hair and subcutaneous fat. The explants are rinsed in sterile PBS, minced finely, and placed in 35-mm Petri dishes containing 200 μ l of Dulbecco's medium supplemented with 20% heat-inactivated fetal calf serum, 50 μ g/ml garamycin, and 5 μ g/ml fungizone. When cells begin to grow, tissue pieces and medium are removed and 2 ml of fresh medium is added. When cells have grown to confluence, they are passaged into 75-cc flasks by rinsing with 1 ml of PBS and incubating with 1 ml of 0.25% trypsin/0.02% EDTA for 5–10 min at room temperature. Subsequent to the initial passage, cells are split at a 1:4 ratio upon reaching confluence. For injection into the vitreous, confluent cells from the third to tenth passage are trypsinized with 0.25% trypsin/0.02% EDTA, centrifuged at 500–600 g, washed twice with the medium, then resuspended in the medium and allowed to stand for 10–15 min to let cell clumps settle. The number of cells in suspension is determined using a Coulter counter. Tuberculin syringes are filled with 600 μ l of cell suspension, which allows five injections in 0.01 ml steps.

Pigmented rabbits of either sex weighing 2–3 kg are anesthetized with 2.0–2.5 ml of a 1:1 Rompun-ketamine mixture. The eyes are dilated using one drop of 10% Neo-Synephrine, 1% Mydracyl, and 1% atropine. Only one eye of each animal is injected using an operating microscope. Fifty thousand, 100,000 or 250,000 cells in a total volume of 0.1 ml medium are injected into the posterior portion of the midvitreous through a 27-gauge needle 5 mm from the corneoscleral limbus at the superonasal quadrant. The test drug or the vehicle is injected as a second injection through the same entrance site and is delivered to the same place in the vitreous. Both injections have to be done slowly to prevent posterior retinal holes or deformation of the injected cells by shearing forces. The needle is withdrawn slowly to prevent leakage of vitreous from the wound. A paracentesis is performed to equilibrate the intraocular pressure after the second injection. An ointment containing antibiotics and atropine is applied at the end of the procedure.

EVALUATION

The development of proliferative vitreoretinopathy in the injected eyes is followed by indirect ophthalmoscopy; the appearance of membranes and detachment is documented by fundus photography.

MODIFICATIONS OF THE METHOD

A survey of eye models of inflammation including conjunctivitis, corneal responses to injurious stimuli, uveitis models, and retinal diseases has been presented by Woodward et al. (1989).

Retinopathy in **rats** resembling lesions seen in human retinopathy of prematurity could be provoked in newborn rats by exposure to 80% O₂ for the first 5 days of life (Ricci et al. 1995, 2000).

Hikichi et al. (1996) described an experimental model of ocular inflammation induced by intravitreal injection of interleukin 1 beta in rabbits.

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0.8

Safety Pharmacology of Drugs with Ophthalmologic Activity

See Schulz (2006).

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Chapter P

Pharmacological Models in Dermatology

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| P.12.4 | Skin Penetration | 1993 | | | |
| P.13 | Biomechanics of Skin | 1994 | PURPOSE AND RATIONALE | | |
| P.13.1 | General Considerations | 1994 | A variety of methods are available for the prospective identification of skin sensitizing chemicals. The methods that have been used are the Draize rabbit irritancy test (Draize et al. 1944; Phillips et al. 1972), the occluded guinea pig patch test (Buehler 1965), and the guinea pig maximization test (Magnusson and Kligman 1969; Magnusson 1980). The guinea pig maximization test is an adjuvant sensitization test requiring intradermal injections of the test substance, in combination with Freund's complete adjuvant, which stimulates non-specifically the immune system of treated animals, enhancing their ability to respond to sensitizing chemicals. | | |
| P.13.2 | In Vitro (ex Vivo) Experiments... | 1995 | PROCEDURE | | |
| P.13.2.1 | Stress-Strain Behavior | 1995 | Groups of 15–20 guinea pigs (Hartley strain) are used. On day 0 an area of 4 × 6 cm over the shoulder region is clipped short with an electric clipper. Three pairs of intradermal injections are made simultaneously, so that on each side of the midline there are two rows of injections each. The injection sites are just within the boundaries of the 2 × 4 cm patch, which is applied one week later. Injections are (1) 0.1 ml Freund's adjuvant alone, (2) 0.1 ml test material, and (3) 0.1 ml test material in Freund's adjuvant. Control animals are given the same injections but without the test agent, i. e. Freund's adjuvant and vehicle. | | |
| P.13.2.1.1 | Measurement of Skin Thickness, Ultimate Load, Tensile Strength, Ultimate Strain and Modulus of Elasticity | 1995 | On day 7, the same area over the shoulder region is again clipped and shaved with an electric razor. The test agent in petrolatum is spread over a 2 × 4 cm filter paper in an even, rather thick layer or, if liquid, to saturation. The patch is covered by an overlapping, impermeable plastic adhesive tape. This in turn is firmly secured by an elastic adhesive bandage, which is wound | | |
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| P.18 | Cutaneous Microcirculation | 2017 | | | |
| P.18.1 | General Considerations | 2017 | | | |

around the torso of the animal. The dressing is left in place for 48 h. Control animals are exposed to the vehicle without the test agent in the same way as the experimental group.

On day 21, in experimental and control animals the flanks are shaved on both sides on an area of 5 × 5 cm each. Filter paper pieces, 2 × 2 cm, are sealed to the flanks for 24 h with the same occlusive bandage as for topical induction: (1) left side: patch with the test agent in the highest non-irritant concentration. The same vehicle as for topical induction is used; (2) right side: patch with the vehicle.

On day 23, reading is made 24 h after removing of the patches. By then, skin irritation due to the occlusive dressing has usually faded.

EVALUATION

If the challenge reactions in the experimental group clearly outweigh those in the control group, the agent is regarded to be a sensitizer. To grade the substances according to the percentage of animals sensitized, the substances are divided into 5 classes, ranging from weak (grade I) to extreme (grade V) sensitizers (Kligman 1966).

MODIFICATIONS OF THE METHOD

Improvement of the classical guinea pig maximization test was proposed by several authors: Maurer et al. (1980a), Shillaker et al. (1989), Botham (1992), Kashima et al. (1993), Basketter et al. (1995), Frankild et al. (1996), Vohr et al. (2000), Steiling et al. (2001).

Maurer et al. (1980b) developed a model for the evaluation of the photocontact allergenic potential in the guinea pig. Four days before the induction period, Pirbright white guinea pigs were shaved on the back skin and chemically depilated. During the 3-week initial induction period, the animals were treated epicutaneously four times a week with the test drug. Four days before the challenge at week 6 the skin was again chemically depilated. During the challenge periods at weeks 6 and 9, the animals were treated for 3 days consecutively with the test drug. To stimulate the immunological reactivity, the guinea pigs received four intradermal injections of Freud adjuvant/saline 1:1. After each application, the animals were irradiated for 15 min with a Osram Xenon 6000 W UV lamp. The intensity of erythematous reactions was evaluated according to the Draize scoring scale.

A predictive assay for contact allergens using human skin explant cultures has been described by Pistoor et al. (1996).

Use of **transgenic animals** to investigate drug hypersensitivity has been proposed by Moser et al. (2001).

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P.1.2**Popliteal Lymph Node Hyperplasia Assay****PURPOSE AND RATIONALE**

The popliteal lymph node assay in mice or rats has been recommended as a tool for predicting allergies (Kammuller et al. 1989; Bloksma et al. 1995; Koch et al. 2000; Pieters 2001). Moreover, the inhibition of popliteal lymph node hyperplasia can be measured (Schorlemmer et al. 1998; Mollison et al. 1999). The test can be used to study compounds potentially effective in allergic eczema

PROCEDURE

Spleens from Brown-Norway rats are harvested aseptically, splenocytes expressed by compression with a hemostat in Dulbecco's phosphate buffered saline (DPBS), red blood cells lysed with Tris (0.16M) buffered in ammonium chloride (0.17M) buffer, washed twice (400 g), irradiated (20 Gy), washed in DPBS, and suspended in DPBS at 5×10^7 cells per ml. On day 0, recipient Lewis rats are injected subcutaneously into the plantar surface of the right hindpaw with 0.1 ml of the splenocyte suspension. Compounds are dissolved in an appropriate vehicle and dosed daily, 2 ml/kg, on days 0–3. Recipients are sacrificed on day 4 and popliteal lymph nodes (PLN) from both hind limbs from vehicle control rats, or the right popliteal lymph node from drug-treated rats, are dissected free and weighed individually on a microbalance (Mollison et al. 1993). The average weight of PLN from the left leg of vehicle-treated animals is used as background.

EVALUATION

Percent inhibition is calculated using the following formula:

$$100 - \frac{PLN_{\text{exp.}} - PLN_{\text{mean left}}}{PLN_{\text{mean right}} - PLN_{\text{mean left}}} \times 100$$

$PLN_{\text{exp.}}$ = experimental PLN weight

$PLN_{\text{mean left}}$ = mean vehicle control left PLN weight

$PLN_{\text{mean right}}$ = mean vehicle control right PLN weight

ED_{50} values are derived by simultaneous least square regressive analysis.

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P.1.3**Local Lymph Node Assay****PURPOSE AND RATIONALE**

More recently, the local lymph node assay in mice has been recommended for measurement of allergenic potency (Kimber and Weisenberger 1989; Kimber et al. 1995; Kimber 2001; Basketter and Scholes 1992; Basketter et al. 2001).

PROCEDURE

Groups of mice (CBA strain), weighing 20–25 g, receive topical applications of the test chemical on the dorsum of both ears, once a day for three consecutive days. In standard analyses, three concentrations of the test material are evaluated together with the relevant vehicle control. Five days after the initiation of exposure, all mice receive an intravenous injection of [^3H]-labeled thymidine into their tail vein. Five hours later, animals are sacrificed and draining auricular lymph nodes excised. A single cell suspension of lymph node cells is prepared by gentle mechanical disaggregation and the cells are washed and resuspended in trichloroacetic acid for at least 12 h at 4°C. Precipitates are resuspended in trichloroacetic acid and transferred to an appropriate scintillation fluid. The incorporation by draining lymph node cells of [^3H]-labeled thymidine is measured by β -scintillation counting and recorded as mean disintegrations per min (dpm).

EVALUATION

For each concentration of the test material a stimulation index (*SI*) is derived relative to the concurrent vehicle control. Those chemicals that at one or more test concentrations induce a *SI* of three or greater are classified as skin sensitizers.

Dose-response curves are plotted in order to provide information on the relative potencies of skin sensitizers. The concentration of the test chemical required to produce a stimulation index (*SI*) of three (named *EC3* value) is calculated using the formula:

$$EC3 = c + [(3 - d)/(b - d) \times (a - c)]$$

where the data points lying immediately above and below the *SI* value of three on the dose-response plot have the coordinates (*a,b*) and (*c,d*), respectively.

MODIFICATIONS OF THE METHOD

On the basis of a modified local lymph node assay, Homey et al. (1997) analyzed immunosuppressive effects of topically applied drugs. On four consecutive days, NMRI mice were treated on the dorsal surfaces of both ears with increasing concentrations of test compound. During the last three days, the mice received in addition the contact sensitizer, oxazolone (1%). On day 5, draining auricular lymph nodes were removed in order to assess lymph node cell counts and perform flow cytometric analysis of lymph cell subpopulations.

Hariya et al. (1998) developed a non-radioactive endpoint in a modified local lymph node assay.

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P.2 Experimental Dermatitis

Several methods involving skin reactions are discussed in other chapters, such as:

- Ultraviolet erythema in guinea pigs (H.3.2.2.1),
- Vascular permeability (H.3.2.2.2),
- Oxazolone-induced ear edema (H.3.2.2.4),
- Croton-oil ear edema in rats and mice (H.3.2.2.5),
- Spontaneous autoimmune diseases in animals (I.2.2.1),
- Passive cutaneous anaphylaxis (I.2.2.4),
- Arthus type immediate hypersensitivity (I.2.2.5),
- Delayed type hypersensitivity (I.2.2.6),
- Reversed passive Arthus reaction (I.2.2.7),
- Acute graft versus host disease (I.2.2.15),
- SLE-like disorder in MRL/lpr mice (I.2.2.16),
- Inhibition of allogenic transplant rejection (I.2.2.20).

P.2.1

Spontaneous Dermatitis

P.2.1.1

NC/Nga Mouse as Model for Atopic Dermatitis

The NC/Nga mouse has been recommended as a model for atopic dermatitis (Matsuda et al. 1997; Tsudzuki et al. 1997; Suto et al. 1999; Vestergaard et al. 1999, 2000; Kotani et al. 2000; Aioi et al. 2001; Kohara et al. 2001). When kept in specific pathogen free conditions, it remains healthy, but when kept in non-sterile conditions, it spontaneously develops a disease resembling atopic dermatitis at the age of 6–7 weeks. The level of IgE in the blood gradually increases to very high levels, and peaks at the age of 16–18 weeks (Matsuda et al. 1997). This enhanced IgE production has been attributed to an increased sensitivity of the B cell to the CD40 ligand and to IL-4, which is the result of enhanced phosphorylation of Janus kinase 3, a feature found also in atopic dermatitis (Matsumoto et al. 1999). At 16–18 weeks, the mice develop dry skin, and, gradually, nodular lesions, which in turn become crusted wounds. The lesions are pruritic and they are located on the back, the neck, the ears and the face. Biophysical parameters show impairment of water retention and barrier function. The amount of ceramide in the skin decreases significantly (Aioi et al. 2001).

Histologically, the skin lesions in the NC/Nga mice are characterized by hyperkeratosis and parakeratosis, which resemble the lichenified lesions observed in atopic dermatitis patients. In the dermis, an infiltration is found, similar to that seen in atopic dermatitis patients, containing lymphocytes, eosinophils, mast cells and macrophages, in addition to a large population of dendritic cells (Vestergaard et al. 1999).

The lesions in the NC/Nga mouse improve after treatment with tacrolimus hydrate (FK506) ointment (Hiroi et al. 1998) and also with topical steroids (Vestergaard et al. 1999). Both treatments reverse the changes in the skin, block the expression of inflammatory cytokines and decrease the serum levels of IgE.

FURTHER DERMATITIS MODELS

Iwasaki et al. (2001) recommended atopic NC/Nga mice as a model for allergic asthma: after immunization with ovalbumin, severe allergic responses were elicited by a single intranasal challenge.

Arai et al. (2004) showed that a prostanoid DP₁ receptor agonist inhibits the pruritic activity in NC/Nga mice with atopic dermatitis.

Mihara et al. (2004) described the vital role of the itch-scratch response in development of spontaneous dermatitis in NC/Nga mice. Capsaicin-sensitive sensory nerves of these mice were ablated by neonatal capsaicin treatment. Scratching behavior was almost completely prevented in these mice and the development of dermatitis and elevation of the serum IgE level were significantly suppressed.

Ohmura et al. (2004) studied the involvement of substance P in scratching behavior in a picrylchloride-induced dermatitis model in NC/Nga mice.

Takano et al. (2004) evaluated the antipruritic effects of several agents on scratching behavior by NC/Nga mice.

Takaoka et al. (2005, 2006) tested the expression of IL-31 gene transcripts in NC/Nga mice with atopic dermatitis. The expression of IL-31 mRNA in the skin of NC/Nga mice with scratching behavior was significantly higher than that in NC/Nga mice without scratching behavior.

Further animal models for atopic dermatitis were described, such as the NOA (Naruto Research Institute Otsuka Atrichia) mouse (Natori et al. 1999; Watanabe et al. 1999). The mice develop ulcerative skin lesions with accumulation of mast cells and increased serum IgE.

A spontaneous mutation characterized by chronic proliferative dermatitis in C57BL mice was described by HogenEsch et al. (1993).

Barton et al. (2000) reported that mice lacking the transcription factor ReIB develop T cell-dependent skin lesions similar to human atopic dermatitis.

Herz et al. (1998) developed a human-SCID mouse model to analyze the possible role of bacterial superantigens in human allergic immune responses under *in vivo* conditions.

Hossen et al. (2005) described the effect of loratadine on atopic-dermatitis-associated pruritus in ICR and hairless mice.

Sonkoly et al. (2006) described IL-31 as a new link between T cells and pruritus in atopic skin inflammation.

Chan et al. (2001) reported that the expression of IL-4 in the epidermis of **transgenic mice** results in a pruritic inflammatory skin disease. The mice spontaneously develop a skin disease reproducing all key features of human atopic dermatitis, including xerosis, conjunctivitis, inflammatory skin lesions, *Staphylococcus aureus* infection, histopathology of chronic dermatitis with T cell, mast cell, macrophage-like mononuclear cell, and eosinophil infiltration, and elevation of total serum IgE and IgG1.

Konishi et al. (2002) demonstrated in IL-18-transgenic mice that IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions.

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P.2.1.2

Motheaten Mice

Mice homozygous for the autosomal recessive motheaten (*me*) or the allelic viable motheaten (*me^v*) mutations develop severe and early-age onset of systemic autoimmune and inflammatory disease (Green and Shultz 1975; Shultz et al. 1984; Shultz 1988; Kovarik et al. 1994; Su et al. 1998). These mice show arthritis, patchy dermatitis and hemorrhagic pneumonitis; the latter is considered to be the cause of the early death of *me* and *me^v* mice at the age of 3 and 9 weeks, respectively.

Homozygous *me^v* mice are identified first at the age of 3–4 days by focal depigmentation of the skin, followed by patchy absence of hair and by necrotic lesions on paws, tail and ears.

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P.2.1.3

Spontaneous Erythema in Hairless Rats

The hairless rat (WBN/Kob-*Ht*) is a dominant mutant derived from the Wistar strain. With an incidence rate of about 4% in both male and female animals, at an age of 20 weeks an erythema appears spontaneously

on the dorsal skin, gradually becoming widespread and progressive in nature (Iwamoto et al. 1997; Tani et al. 1998). Histopathologically, erythema is characterized by dermatitis induced by an immunological reaction. Areas of erythema in the skin were decreased by treatment with dexamethasone (1 mg/kg) or cyclosporine (25 or 50 mg/kg) injected subcutaneously every other day for 2 weeks. The results suggested that erythema on the hairless rat could be used as an animal model of spontaneous dermatitis.

MODIFICATIONS OF THE METHOD

Atopic dermatitis-like symptoms were reported in hypomagnesaemic hairless rats by Chavaz et al. (1984) and Neckermann et al. (2000).

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P.2.1.4

Spontaneous Atopic Dermatitis in Dogs

PURPOSE AND RATIONALE

Atopic dermatitis is a known chronic inflammatory skin disease in dogs (Scott et al. 2001).

Olivry et al. (2001) characterized the inflammatory infiltrate during IgE-mediated late phase reactions in the skin of normal and atopic dogs with *Dematophagoides farinae*-induced allergy.

Nuttall et al. (2002) studied the expression of Th1, Th2 and immunosuppressive gene transcripts in canine atopic dermatitis and found over-production of IL-4. Canine atopic dermatitis was proposed as a possible animal model of human atopic dermatitis.

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P.2.2

Contact Dermatitis

P.2.2.1

Contact Hypersensitivity in Animals

PURPOSE AND RATIONALE

The phenomenon of contact hypersensitivity in animals is thought to mirror atopic dermatitis or eczema in patients (Corsini et al. 1979; Cooper 1994; Leung 1997).

P.2.2.1.1

Contact Hypersensitivity in Mice

PROCEDURE

CD1 mice are sensitized on the shaved abdomen with 20 µl of 0.5% 2,4-dinitrofluorobenzene (DNFB) in a vehicle consisting of 95% acetone/5% olive oil on days 0 and 1. On day 5, groups of 10 mice are challenged with 0.2% DNFB with or without co-dissolved drug, 10 µl on both the internal and external surface of both ears. Mice are sacrificed 24 h post-challenge, and a 7 mm diameter circle punched from each ear is weighed immediately on a microbalance.

EVALUATION

The mean earplug weight from naïve mice challenged with 0.2% DNFB are used as background control.

Percent inhibition is calculated using the following formula:

$$100 - \frac{\text{plug}_{\text{exp.}} - \text{plug}_{\text{mean naïve}}}{\text{plug}_{\text{mean sens.}} - \text{plug}_{\text{mean naïve}}} \times 100$$

plug_{exp.} = experimental ear plug weight

plug_{mean naïve} = mean naïve ear plug weight

plug_{mean sens.} = mean sensitized vehicle control plug weight

*ED*₅₀ values are derived by simultaneous least square regressive analysis.

MODIFICATIONS OF THE METHOD

Lowe et al. (1977) used oxazolone-sensitized Swiss Webster mice to evaluate anti-inflammatory properties

of a prostaglandin antagonist, a corticosteroid and indomethacin in experimental contact dermatitis.

Studies in mice on allergic contact dermatitis were performed by Ek and Theodorsson (1990), Friginals et al. (1990), Katayama et al. (1990), Lavaud et al. (1991), Trenam et al. (1991), Stanley et al. (1991), Maguire (1996), and Corsini and Galli (1998).

Grabbe et al. (1995) showed that removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of contact hypersensitivity in BALB/c mice.

Koyama et al. (1998) used tenascin-C knockout mice and studied the effect on dinitrofluorobenzene-induced dermatitis.

Meingassner et al. (1997) sensitized female NMRI mice on the shaved abdomen with 50 μ l of oxazolone (2% in acetone). After 7 days, they were challenged with 10 μ l of 2% (for topical testing) or 0.5% (for systemic testing) oxazolone on the inner surface of the right ear. Compounds were tested topically by a single application of 10 μ l ethanolic solution to the challenge site, 30 min after challenge. Compounds were tested for systemic activity by administration of a single subcutaneous injection immediately after challenge or by two oral doses (2 h before and immediately after challenge). The unchallenged left ears served as normal controls. Dermatitis was evaluated from the difference in pinnal weight, which was taken as a measure of inflammatory edema 24 h after challenge.

Traidl et al. (1999) reported inhibition of allergic contact dermatitis to dinitrochlorobenzene (DNCB) but not to oxazolone in interleukin-4-deficient mice.

Morita et al. (1999) reported that fur mites induce dermatitis associated with IgE hyperproduction in NC/Kuj mice. Four-week-old NC/Kuj mice were kept together with NC mice infected with fur mite (*Myocoptes musculinus*) for 2 weeks in isolated clean rooms and thereafter separated. Serum IgE levels were determined and the skin examined histologically.

Matsuoka et al. (2001) described a mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. The mites were cultured in a mixture of mouse diet and dried yeast in culture flasks. A crude extract in PBS was applied to the shaved back of NC/Nga mice or BALB/c mice three times a week for 8 weeks. In the NC/Nga group, dryness and scaling appeared on the skin, and scratching behavior increased at the second week in the treated group. Skin erosion and hemorrhage occurred in the fourth week. The epidermis thickened and deepened into the upper dermis, in which mast cells were highly

accumulated, corresponding with the skin lesions of atopic eczema/dermatitis syndrome in patients.

Matsui and Nishikawa (2002) used female specific-pathogen-free BALB/c mice at an age of 6–8 weeks. The animals were barrier-disrupted by repeated applications of adhesive cellophane tape prior to percutaneous sensitization by topical administration of 100 μ l of a solution of house dust mite antigen. Seven days after the sensitization, the mice were challenged on the shaved dorsal skin near the ear by subcutaneous injection of 10 μ l of mite antigen or lipoteichoic acid from *Staphylococcus aureus* for elicitation of localized skin inflammation. The cytokine response in the dorsal skin was investigated by reverse transcription-polymerase chain reaction and immunohistological analysis.

Spergel et al. (1998) reported that epicutaneous sensitization with ovalbumin induced localized allergic dermatitis and hyper-responsiveness to methacholine after single exposure to aerosolized antigen in mice.

Spergel et al. (1999) used mice with targeted deletions of IL-4, IL-5, and interferon- γ cytokine genes to assess the role of these cytokines in a murine model of allergic dermatitis elicited by epicutaneous sensitization with ovalbumin.

Laouini et al. (2003) used IL-10^{-/-} mice to examine the role of IL-10 in a mouse model of allergic dermatitis induced by epicutaneous sensitization with ovalbumin on tape-stripped skin.

Takeshita et al. (2004) underlined the essential role of MHC II-independent CD4⁺ T cells, IL-4 and STAT6 in contact hypersensitivity induced by fluorescein isothiocyanate in the mouse and recommended fluorescein-isothiocyanate-induced contact hypersensitivity as a suitable animal model for atopic dermatitis.

Sasakawa et al. (2001) induced atopic dermatitis-like lesions by topical application of mite antigens in NC/Nga mice. Extracts of mite antigen were injected repeatedly at the ventral side of the ear. Clinical symptoms and thickness of the ear were measured. On day 18, blood and submandibular lymph nodes were collected to measure plasma immunoglobulins and to perform histochemical analysis.

Heishi et al. (2003) performed gene expression analysis of atopic dermatitis-like skin lesions induced in NC/Nga mice by mite antigen stimulation under specific pathogen-free conditions. Mite Extract-Dp (LSL Japan) was injected intradermally into the right and left pinnae and into the skin of NC/Nga of BALB/c mice in two places once per 3 days, and the clinical symptoms and the ear thickness were measured. On day 14 or 28, plasma IgE was determined. Further-

more, mRNA transcripts in pinnae were analyzed using real-time quantitative PCR.

Food hypersensitivity plays a pathogenic role in patients with atopic dermatitis (Sicherer and Sampson 1999).

Li et al. (2001) reported a murine model of atopic dermatitis associated with food hypersensitivity. Female C3H/HeJ mice were sensitized orally to cow's milk or peanut with cholera toxin adjuvant and then subjected to low-grade allergen exposure. Histologic examination of skin lesions, allergen-specific Ig levels, and allergen-induced T-cell proliferation were studied. Treatment of the eruption with topical corticosteroids led to decreased pruritus and resolution of the cutaneous eruption.

P.2.2.1.2

Contact Hypersensitivity in Rats

Meingassner et al. (1997) sensitized female Sprague Dawley rats by application of 80 μ l of 2% 2,4-dinitrofluorobenzene (DNFB, dissolved in acetone, DMSO and olive oil 50:10:38 v/v/v), applied in 20 μ l volumes to the inner surface of both ear lobes and to both shaved inguinal regions on day 1. Allergic contact dermatitis was elicited with 30 μ l of 0.5% DNFB applied to the test sites of \approx 15 mm in diameter on both shaved flanks on day 12. Animals were treated twice by gavage 2 h before and immediately after challenge. Dermatitis was evaluated by measuring the thickness of the lifted skin fold at the test sites with a spring-loaded micrometer (Schnelltaster, Kröplin, Germany) before challenge and 24 h after challenge.

P.2.2.1.3

Contact Hypersensitivity in Guinea Pigs

Guinea pigs, six per group, are sensitized on the dorsal surface of one ear pinna with 50 μ l of 10% DBFB in 50% acetone: 50% olive oil on day 0, and then on the opposing ear on day 1. On day 5, animals are shaved and challenged with 0.5% 1-chloro-2,4-dinitrochlorobenzene (DNFB) with or without co-dissolved drug, 15 μ l per site, 4.8 μ l per cm², on the dorsolateral surface (Hsieh et al. 1996). Naïve animals are challenged with DNFB and serve as non-specific controls. The response is scored visually in a blinded fashion 24 h after challenge (0: no change; 0.5: questionable erythema; 1: faint or scattered erythema; 2: mild, confluent erythema; 3: moderate erythema without edema or induration; 4: strong erythema with uniform induration or edema; 5: severe erythema

with induration or edema, plus ulceration). Data are calculated as for mouse contact hypersensitivity.

Rosenqvist et al. (1991) studied the effects of cilazaprilat and enalaprilat on experimental dermatitis in guinea pigs.

Boyera et al. (1992) tested repeated application of dinitrochlorobenzene to the ears of sensitized guinea pigs as an animal model for contact eczema in humans.

The effect of a topical preparation of mycophenolic acid on experimental allergic contact dermatitis of guinea pigs induced by dinitrofluorobenzene was described by Shoji et al. (1994).

P.2.2.1.4

Contact Hypersensitivity in Pigs

Groups of 6–12 pigs are sensitized with 10% DNFB in acetone/DMSO/olive oil (45:5:50 by volume) to the shaved outer aspect of both ears and bilateral sites of the lower abdomen, 100 μ l per site, on day 0, with a second application of 5% DNFB to the internal pinna and the lower thorax on day 3. On day 9, pigs are restrained on a webbed canvas cart and the test area carefully shaved with an electric clipper. A pilot challenge with 0.1, 0.15, and 0.2% DNCB in acetone/olive oil (95:5 by volume), 3.8 μ l per cm², is used to determine conditions for obtaining submaximal average response in each animal cohort. Pigs are scored by a blinded observer, 24 h post-challenge on a scale from 0 to 4 (0: no change; 0.5: questionable erythema; 1: faint or scattered erythema; 2: moderate erythema without induration or edema; 3: strong erythema with focal areas of edema or induration; 4: extreme erythema with uniform induration or edema). Pigs having a mean DNCB control score <1.5 are excluded (Hsieh et al. 1997). Based on their pilot response on day 10, animals are stratified into two groups having comparable mean scores, challenged on duplicate sites with DNCB in 95:5, with or without co-dissolved drug, and scored on day 11. Scores for each challenge site are compared with the average of the control spots treated with DNCB alone on the same pig, and expressed as percentage inhibition.

Bilski and Thomson (1984) recommended allergic contact dermatitis in the domestic pig as a model for evaluating the topical anti-inflammatory activity of drugs and their formulations.

Meingassner and Stütz (1992) sensitized female domestic pigs with a 10% solution of DNFB dissolved in 50% acetone, 10% dimethylsulfoxide (DMSO) and 30% olive oil applied in volumes of 100 μ l onto both auricles (medial aspects) and groins on day 1. The ani-

mals were exposed again on the lateral aspects of both auricles to 100 µl of 2% DNFB on day 4. Challenge was performed with a 1% DNFB solution (without DMSO) on day 12 by applying 20 µl epicutaneously to each of 24 test sites (2 cm diameter) arranged in four craniocaudal lines on the dorsolateral back of each animal. The test sites were treated twice (0.5 and 6 h after challenge) with 20 µl of solutions of active compound or drug vehicle. Evaluation of the treatment-related effects was performed at the peak inflammatory response which was 24 h after challenge. Each test site was evaluated visually for (1) intensity, (2) extent of erythema, and (3) consistency using arbitrary scores from 0 to 4. In addition, skin changes were biophysically characterized by measuring microvascular perfusion (PeriFlux PF3 Laser Doppler Perfusion Monitor) and reflective color measurement (Minolta Chroma Meter CR 200).

Vana and Meingassner (2000) described morphologic and immunohistochemical features of experimentally induced contact dermatitis in **Göttingen minipigs**.

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P.2.2.2

Non-Immunologic Contact Urticaria

PURPOSE AND RATIONALE

Contact urticaria is defined as a wheal-and flare reaction, appearing shortly after certain substances contact intact skin and disappearing within some hours, leaving normal appearing skin. Contact urticaria is divided into two main types, immunologic and non-immunologic (Maibach and Johnson 1975). Immunologic contact urticaria is mediated at least partially by specific IgE antibodies attached to mast cell membranes. Vasoactive substances, released from mast cells, elicit erythema and edema of the skin. Non-immunologic contact urticaria appears only on the contact area without previous sensitization. Specific antibodies against the causative substance are not found in serum. Lahti and Maibach (1984) investigated the suitability of the guinea pig for studies on non-immunologic contact urticaria.

PROCEDURE

Female Hartley strain guinea pigs weighing 350–500 g are used. Fifty μ l of test substances is applied with a micropipette to both sides of the earlobe. One ear of the animal is challenged with the contact urticant, while the other ear serves as control with ethyl alco-

hol. Earlobe thickness is measured three times on four different sites using a string micrometer with round touching plates, 6 mm in diameter. The string of the instrument is adjusted so that the moving plate does not squeeze the tissue, but stops at once when it reaches the surface of the ear. The mean of 12 measurements is recorded as the pre-application thickness. All measurements after application of the test substance are performed once on the same four sites, and the mean is recorded as the post-application thickness.

The thickness of the ear is measured 5 min after application, and then every 10 min during the first h, every 15 min during the second, and every 30 min during the third h.

EVALUATION

The differences between post-application and pre-application values are recorded and plotted as time-response and dose-response curves.

MODIFICATIONS OF THE METHOD

Lauerma et al. (1997) used the trimellitic anhydride-sensitive mouse as an animal model for contact urticaria. BALB/c mice were sensitized with trimellitic anhydride by topical applications and treated with glucocorticosteroids, antihistaminics, or non-steroidal anti-inflammatory drugs. Ears were challenged with trimellitic anhydride and ear thickness was measured at baseline and 1, 2, 4, 8, and 24 h after challenge. Trimellitic anhydride caused a biphasic ear swelling response. However, there was also an early swelling by trimellitic anhydride in non-sensitized mice, suggesting that non-immunological as well as immunological mechanisms contribute to early swelling by trimellitic anhydride.

Irritant contact dermatitis was induced in mice by the application of 20 μ l 0.01% A23187 (calcium ionophore) or 0.005% PMA (phenyl mercuric acetate) to both aspects of the right pinnae which were treated simultaneously (A23187) or after 30 min (PMA) with the test compound or vehicle (Meingassner and Stütz 1992). Topical efficacy was assessed by determination of the differences in weight of both auricles of treated and untreated mice 7.5 h (A23187) or 6 h (PMA) after application of the irritants.

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P.2.3

Immunological Models of Atopic Dermatitis

P.2.3.1

Mononuclear Cells from Atopic Dermatitis Donors

PURPOSE AND RATIONALE

Rühl et al. (2003) studied the effects of PPAR- α and PPAR- β ligands on immunoglobulin synthesis and cytokine production by peripheral blood mononuclear cells from normal donors and patients with atopic dermatitis.

PROCEDURE

Peripheral blood mononuclear cells were isolated from buffy coats of non-allergic healthy donors and atopic dermatitis patients by Ficoll Hypaque (D = 1.077) separation (350 g, 30 min, room temperature). B cells were purified by magnetic cell sorting using anti-CD19-coupled magnetic beads. Then 4×10^8 peripheral blood mononuclear cells in 800 μ l PBS + 20 nM ethylenediaminetetraacetic acid + 0.2% bovine serum albumin + 15 μ l Beriglobin (Chinon Bering, Marburg, Germany) were incubated for 10 min in ice, followed by addition of 200 μ l conjugated beads (1.5 ratio) and 10 min of incubation at 4°C. After several washings, CD19-positive B cells were selected by magnetic positive selection with LS⁺ column, and the cells were then resuspended in medium and counted. The same procedure was done for the purification of T cells and monocytes, respectively, using anti-CD3 and anti-CD14 coupled magnetic beads.

Peripheral blood mononuclear cells and purified B cells (10^6 cells per ml) were cultured in RPMI 1640 culture medium supplemented with L-glutamine (2 mM), penicillin (100 U per ml), streptomycin (100 μ g/ml) and 10% heat inactivated fetal calf serum. All cell cultures were carried out at 37°C in humidified air and 5% CO₂ atmosphere.

For the immunoglobulin assays, immunoplates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with the isotope-specific anti-human Ig-Fc antibodies diluted in 0.1 M bicarbonate buffer. Blocking was performed by adding 2% bovine serum albumin/Tris-buffered saline for 1 h, followed by several washings. Supernatants and internal standard were then added to the wells and incubated in duplicate for

2 h. After several washes, the alkaline phosphatase-conjugated antibodies (IgA, IgG and IgM) or biotinylated anti-IgE MoAb was added. For IgE, plates were incubated for another hour with alkaline-phosphatase-conjugated streptavidin. Following the final reaction with phosphatase substrate (Sigma, Dreieich, Germany), plates were read in a microplate ELISA reader at 405 nm, and the amount of immunoglobulin was calculated according to the standard curve. Cytokines were determined by ELISA assays.

EVALUATION

Wilcoxon's signed rank test for matched pairs was performed to compare the results within the donor groups.

MODIFICATIONS OF THE METHOD

Furthermore, Rühl et al. (2003) performed *in vivo* experiments in ovalbumin-sensitized female NMRI mice confirming the *in vitro* findings and showing that the IL-4-mediated immune response was inhibited in PPAR-activator-treated mice.

Sperhake et al. (1998) studied the effects of recombinant human soluble interleukin-4 receptor on interleukin-4/staphylococcal enterotoxin B-stimulated peripheral mononuclear cells from patients with atopic eczema.

Hong et al. (2003) demonstrated the PPAR γ -dependent anti-inflammatory action of rosiglitazone in human monocytes. Furthermore, it was shown that the suppression of tumor necrosis factor α (TNF α) secretion is not mediated by phosphatase and tensin homolog (PTEN) regulation.

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P.2.3.2

The SCID-hu Skin Mouse as Model for Atopic Dermatitis

PURPOSE AND RATIONALE

Severe combined immunodeficiency (SCID) mice have a defect in the antigen recombinant system that prevents the development of mature B and T lymphocytes (Bosma et al. 1983) and therefore renders these mice tolerant to xenografts of human cells and tissues.

Carballido et al. (2000, 2003), Biedermann et al. (2002, 2004), Igney et al. (2004) and Lametschwandner et al. (2004) reported the establishment of an *in vivo* mouse model that allows monitoring of human T cell migration into human skin. This model is based on the use of SCID mice transplanted with human skin (SCID-hu Skin mice). Adoptively transferred human T helper (Th)2 cells obtained from atopic dermatitis skin lesions or peripheral blood T cells selectively migrate to the human skin grafts of these SCID mice in response to defined chemokines locally injected in the human skin grafts. Homing of human T cells into the human skin on SCID-hu Skin mice is a specific process since it only occurs in response to chemokine ligands that are specific for the chemokine receptors expressed on the migrating T cells.

PROCEDURE

Generation of SCID-hu Skin Mice

Human adult skin is obtained from breast or abdominal reduction surgery. The skin is perfused with cell culture media containing penicillin and streptomycin and fat tissues are removed. Subsequently, full thickness skin is trimmed at the dermal side to obtain skin biopsy samples containing the full epidermis, the papillary dermis, and part of the reticular dermis. This skin tissue is cut into pieces of approximately 10 × 10 mm and kept at 4°C in cell culture media until use. C.B-17/GbmsTac-*Prkdc*^{scid} *Lysf*^{bg} mice (M&B, Ry, Denmark, herein referred to as SCID mice) are anesthetized by i.p. injection of a solution containing ketamine and xylazine and their backs shaved. Subsequently, two pieces of skin of the same size as the human grafts are removed from the back of the mice and replaced by human skin pieces, which are fixed at their edges using surgical glue. At the end of the procedure, the human grafts are covered with a polyvinyl bandage to prevent scratching and to facilitate tissue engraftment. After transplantation, the human grafts undergo a period of human keratinocyte hyperproliferation resulting in the formation of hyperkeratotic crusts. During this process, human skin grafts fuse with the adjacent mouse skin. About 5 weeks after transplantation, the crusts fall off and reveal a skin tissue containing all the characteristic structures of normal human skin including a network of newly grown vessels connecting the graft with the underlying mouse tissues.

Generation and characterization of human T cells for skin homing studies *in vivo*

Th lymphocytes selected for *in vivo* migration studies in SCID-hu Skin mice are isolated from human atopic

skin lesions in order to obtain Th cells with optimal skin homing potential. Human Th cell lines are grown from biopsies of **atopic dermatitis** patients challenged with house dust mite (HDM) antigens (Carballido et al. 1997). Th cell clones are isolated from the HDM-specific lines by limiting dilution and maintained by repeated stimulation with irradiated allogenic human peripheral blood mononuclear cells (PBMC) and phytohaem agglutinin (PHA), followed by expansion with human IL-2.

Atopic dermatitis is an inflammatory skin disease in which skin-infiltrating allergen-specific Th2 cells play a crucial role (Robert and Kupper 1999). Accordingly, the majority of the human Th cell clones isolated from atopic skin biopsy samples are of the polarized Th2 phenotype. These cells produce large amounts of IL-4, IL-5 and IL-13 but low or undetectable levels of IL-2 and IFN- γ . All Th cells express CLA LFA-1 and CCR4. Based on these results and the observation that CCL22 (macrophage-derived chemokine, MDC) is abundantly produced in atopic dermatitis skin lesions, CCL22 is selected as a relevant ligand for the evaluation of skin-specific migration of human Th cells in the SCID-hu Skin mouse model. In order to demonstrate that CCL22 induces Th2 cell activation, Th2 clones are loaded with Fluo-4acetoxymethyl ester (Fluo-4/AM, Molecular Probes, Eugene, Ore., USA). Following the addition of CCL22 (300 ng/ml), changes in fluorescence intensity are analyzed every 5 s using a FACScan FL-1 channel (Becton Dickinson, San Jose, Calif., USA) (Biedermann et al. 2002). Comparable results were obtained with CCL17 (thymus-activation-regulated chemokine, TARC), which is the other ligand for CCR4 and is also abundantly expressed in atopic dermatitis lesions. These results demonstrate the relevance of CCL17 and CCL22 interactions with CCR4 for activation of Th2 cells derived from atopic dermatitis skin lesions. In addition, human peripheral T cells are analyzed for their skin homing capacity in SCID-hu Skin mice. PBMC are isolated from healthy volunteers.

Recruitment of Human Th Cells to Human Skin Grafts in SCID-hu Skin Mice

To evaluate the potential of selected chemokines to recruit atopic-dermatitis-derived human Th2 cells and peripheral blood T cells to human skin *in vivo*, 1.5×10^8 Th2 cells or PBMC are adoptively transferred (i.p.) into SCID mice which have been previously transplanted (6–8 weeks) with two pieces of human skin (SCID-hu Skin mice). Following Th cell transfer, 300 ng of CCL22, CXCL10 (IFN- γ -inducible

protein of 10 kDa, IP-10), or vehicle control is injected intradermally (i.d.) in the human skin grafts of the SCID-hu Skin mice. On day 8, mice are sacrificed and human skin grafts are harvested and processed into single-cell suspensions using a mechanical tissue disaggregator. These cell suspensions are stained with human anti-CD3, anti-CD4 or anti-CD45 mAb conjugated to FITC and PE (Becton Dickinson) and analyzed in the FACScan. CCL22 effectively recruits human Th2 cells into human skin grafts of SCID-hu Skin mice, whereas CXCL10 is ineffective. These data are consistent with the expression of CCR4 on atopic-dermatitis-derived Th2 cells, whereas CXCR3, which is the receptor for CXCL10, is not expressed on these cells. Furthermore, treatment of Th2 cells prior transfer into SCID mice with *Bordetella pertussis* toxin, an inhibitor of G-protein-coupled receptors, completely abolishes the migratory capacity of these Th2 cells indicating the requirement of an intact chemokine receptor signaling pathway (Biedermann et al. 2002). It has to be noted that CCR4-mediated human skin homing of Th2 cells is not restricted to CCL22 since the alternative CCR4-ligand, CCL17, also induces Th2 migration to human skin grafts, although to a lesser degree than CCL22.

Similarly to Th2 cells, T cells present in human PBMC can also be recruited into human skin grafts of SCID-hu Skin mice. These T cells migrate in response to both CCL22 and CXCL10 chemokines (300 ng injected i.d. into human skin), which is consistent with CCR4 and CXCR3 expression on these cells. Peripheral blood T cells also express functional CCR2, since the T cells migrate into human skin in response to the CCR2 ligand CCL2. From these data it can be concluded that both skin-derived Th2 cells and peripheral blood T cells can be recruited into human skin grafts provided that these T cells express the chemokine receptors specific for the recruiting chemokine.

Inhibition of Human T Cell Migration into Human Skin of SCID-hu Skin Mice

The present SCID-hu Skin mouse model has been validated for measuring various inhibitors of human T cell rolling, extravasation and migration *in vivo*. It was shown that inhibition of CLA/E-Selectin interactions using an E-Selectin-specific mAb or a low molecular weight (LMW) E-Selectin-specific antagonist strongly inhibited CCL22-mediated Th2 cell migration into the skin (Biedermann et al. 2002). Similarly, anti-LFA-1 mAbs administered at 5 mg/kg at day 1 prior to Th2 cell transfer and on day 4 strongly inhibits Th2 cell migration into the human skin. Th2 cell migration in

response to CCL22 is also inhibited in a dose-dependent fashion by LMW LFA-1 inhibitors, given daily p.o. starting on the day of T cell transfer.

CRITICAL ASSESSMENT OF THE METHOD

This mechanistic model allows analysis of the relevant steps involved in human T-lymphocyte migration into inflamed skin. In addition, it can be used for preclinical testing of drug candidates that are highly selective for human target molecules associated with the different steps of T cell migration in an environment that resembles the physiologic or pathologic conditions occurring in humans. The model allows the study of chemotactic responses of human cells toward specific, intracutaneously administered chemokines and testing of specific antagonists of human Th cell migration *in vivo*.

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P.3 Pruritus Models

P.3.1

Pruritus and Scratching Behavior in Mice

PURPOSE AND RATIONALE

Pruritus is a common feature of many skin disorders. Since its pathogenesis is largely unknown, proper treatment is not available. The development of new treatments is hampered by the lack of animal models for studying pruritus (Woodward et al. 1985). Kuraishi et al. (1995) have proposed that pruritogenic but not algesiogenic agents stimulate the scratching activity in mice. Scratching is usually registered by counting the number of scratches from direct visual observation or from a video recording (Gmerek and Cowan 1983; Larsen et al. 1994; Thomas et al. 1994). Elliott et al. (2000) developed a method, which allows the automated registration of the scratching activity of the hind legs of mice for periods longer than 24 h.

PROCEDURE

Mice with chronic proliferative dermatitis (cpdm/cpdm mice), a spontaneous mutation of C57BL/Ka mice showing a skin disorder accompanied by severe scratching (Gijbels et al. 1996, 2000), at an age of 6–12 weeks or normal C57BL/Ka mice treated subcutaneously with 100 µg/mouse compound 48/80 or 3 mg/mouse histamine hydrochloride, are used.

Metal rings made of soft 1-mm-diameter aluminum wire are placed around both hind legs of the animal just above the ankle. The ring has to be of sufficient diameter that it is just free enough to rotate around the limb. Mice are housed individually in cages (12 × 18 × 13 cm), which are placed on a scratch detection unit consisting of a plastic outer casing containing the circuit board, and, inset into the top, four ferrite rods with copper coils.

As the mouse scratches, the movement of the metal rings (fitted around its hind legs) in the field generated by the coils elicits a signal that can be transformed into peaks of different frequencies using fast Fourier transformation. Data are downloaded to memory every 3 s, filtered and a power spectrum constructed. The power spectrum is analyzed for a scratch pattern every 1.5 s. Scratching is routinely classified as a signal that gives rise to peaks with maximal amplitudes of around 200 mV and frequencies greater than 15 Hz. The amplitude of general motor activity peaks (movement from bedding using hind limbs, drinking from water bottle) is usually greater than 500 mV and at fre-

quencies of less than 10 Hz. In order to obtain the best discrimination between scratching and general motor activity, any contribution to the scratch frequencies of high energy/low frequency peaks is filtered out by setting the upper detection limit at 0.5 mV for scratching.

EVALUATION

Data are expressed as mean ± SEM. Statistical analysis is performed by one-way analysis of variance followed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Inagaki et al. (2002, 2003) used new apparatus, MicroAct, to study mechanisms in the induction of scratching behavior in BALB/c mice by compound 48/80. A small magnet (1 mm in diameter, 3 mm long) was inserted subcutaneously in both hind paws under ether anesthesia. The mouse with magnets was placed in an observation chamber (11 cm in diameter, 18 cm high), which was surrounded by a round coil. The electric current induced in the coil by the movement of magnets attached to the hind paws was amplified and recorded. Characteristic waves reflecting scratching behavior were detected by a computer.

Brash et al. (2005) designed a repetitive movement detector used for automatic monitoring and quantification of scratching in mice. The system is based on a sensitive force transducer positioned below a recording platform holding a lightweight recording box in which the animal is placed. A programmed microcontroller is used to discriminate between non-specific movement, grooming behavior, and scratching movements made by the animal's hind limb. Following subdermal injections of histamine receptor agonists into the neck of a mouse, dose-related scratching occurred which was detected and quantified.

Larsen et al. (1994) studied the influence of ultraviolet irradiation on scratching behavior in hairless mice. Especially the wavelengths 315–330 nm were more itchy provoking than erythemogenic.

Several authors used the model of **compound 48/80-induced scratching behavior** in mice. Sugimoto et al. (1998) studied the effects of histamine H₁ receptor antagonists on compound 48/80-induced scratching behavior in mice. Rojavin et al. (1998) investigated the antipruritic effect of millimeter waves in mice treated with compound 48/80. Inagaki et al. (2002) studied the mechanisms of induction of scratching behavior in BALB/c mice by compound 48/80. Shinmei et al. (2004) studied the effect of Brazilian propolis on scratching behavior induced by compound 48/80 and histamine in mice. Oliveira et al. (2004) re-

ported suppression of the scratching behavior induced by dextran 40 and compound 48/80 by treatment with pentacyclic triterpenoids. Inagaki et al. (1999, 2000) studied the participation of histamine H₁ and H₂ receptors in passive cutaneous anaphylaxis-induced scratching behavior in ICR mice and evaluated anti-scratch properties of drugs in BBLB/c, ICR, and ddY mice treated with dinitrofluorobenzene painting.

Hossen et al. (2003) reported involvement of histamine H₃ receptors in scratching behavior in mast-cell-deficient mice.

The involvement of the μ - and κ -opioid system in scratching behavior was investigated by Tohda et al. (1997), Kamei and Nagase (2001), and Umeuchi et al. (2003, 2005).

Tan-No et al. (2000) reported that intrathecally administered spermine produces scratching, biting, and licking behavior in mice.

Hansson et al. (2002) described a model of chronic itchy dermatitis in transgenic mice with epidermal overexpression of stratum corneum chymotryptic enzyme.

Andoh and Kuraishi (2002) described inhibitory effects of azelastine on the substance P-induced itch-associated response in mice.

Umeuchi et al. (2005) tested spontaneous scratching behavior in MRL/lpr mice, a possible model for pruritus in autoimmune diseases, and antipruritic activity of a κ -opioid receptor agonist and recommended MRL/lpr mice scratching behavior as a suitable model of pruritus that occurs in autoimmune diseases.

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P.3.2

Pruritic Dermatitis in Other Species

Gmerek and Cowan (1983) described an animal model for preclinical screening of systemic antipruritic agents in **rats**. Bombesin induces dose-related excessive scratching when administered intracerebroventricularly (i.c.v.) to rats. Scratching elicited by 0.10 μ g bombesin i.c.v. was monitored with the help of a microcomputer.

Thomas and Hammond (1995) found that microinjections of morphine into the rat medullary dorsal horn produces a dose-dependent increase in facial scratching in Sprague Dawley rats.

Thomsen et al. (2001) proposed scratch induction in the rat by intradermal serotonin as a model of pruritus. Several compounds (histamine, compound 48/80, kallikrein, trypsin, papain, substance P and PAF) were injected into the rostral back of rats. Only serotonin induced excessive scratching at the site of injection. The model was recommended for research and development of antipruritics of the non-histaminic type.

De Castro Costa (1987) tested scratching behavior in arthritic rats; Thomsen et al. (2002), in hairless rats; Ozaki et al. (2005), in beige rats with IgE hyperproduction.

Hayashi et al. (2001) studied the effects of a histamine H₁ antagonist on cutaneous hyperpermeability and scratching behavior induced by poly-L-arginine in rats.

Noshima and Carstens (2003) described quantitative assessment of directed hind limb scratching behavior in Sprague Dawley rats after intradermal injection of serotonin into the nape of the neck.

Gingold and Bergasa (2003) found that a cannabinoid agonist increases nociception threshold in rats with cholestasis secondary to bile duct resection. The model was recommended when testing compounds for treatment of pruritus due to cholestasis.

Minami and Kamei (2004) published a chronic model for evaluating the itching associated with allergic conjunctivitis in rats. After subcutaneous and local sensitization with egg albumin, scratching behavior was observed after instillation of egg albumin into the eyes.

Butler et al. (1983) described pruritic dermatitis in asthmatic basenji-greyhound **dogs**, which was manifested as lichenified plaques and as inflammatory nodules and papules. The authors recommended this disease as a model for human atopic dermatitis.

Osifo (1991) used a dog model to study structure–activity relationships in the pruritogenicity of chloroquine and amodiaquine metabolites.

Patterson and Harris (1981) reported chronic pruritic dermatitis in asthmatic **rhesus monkeys**.

Ko and Naughton (2000) described an experimental itch model in rhesus monkeys.

Holden et al. (2003) found in this model that activation of κ -opioid receptors inhibits pruritus evoked by subcutaneous or intrathecal administration of morphine.

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P.4 Psoriasis Models

P.4.1

General Considerations

Several authors reviewed the experimental models for psoriasis (Krueger and Jorgensen 1990; Boehncke 1997; Nickoloff 1999; Rosenberg et al. 1999; Schön 1999; Mizutani et al. 2003). In addition to studies in animals, various *in vitro* investigations were carried out with autopsy material from human psoriatic lesions. Several studies were devoted to the T-cell hypothesis of psoriasis (Bos and de Rie 1999). Lymphocytes may change epidermal growth homeostasis, leading to increased keratinocyte proliferation and abnormal differentiation of these apoptotic cells that end their life cycle as corneocytes.

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P.4.2

In Vitro Studies with Isolated Cells

P.4.2.1

Cultured Keratinocytes

PURPOSE AND RATIONALE

Cultured keratinocytes have been used to study the pathogenesis of psoriasis (Mils et al. 1994; Bata-Csörgö et al. 1993, 1995a, b; Ockenfels et al. 1996; Nylander-Lundqvist and Egelrud 1997a, b; Nylander-

Lundqvist et al. 1998; Konger et al. 1998; Szabo et al. 1998; Dimon-Gadal et al. 2000; Karvonen et al. 2000; Segaert et al. 2000; Ting et al. 2000) and to evaluate antipsoriatic drugs (Chapman et al. 1990; Ockenfels et al. 1995; Medalie et al. 1996; Lin et al. 1999; Diaz et al. 2000; Farkas et al. 2001). Sampson et al. (2001) tested the *in vitro* keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins.

PROCEDURE

The medium (FDMEM) consists of Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 2% streptomycin.

SVK-14 keratinocytes (Taylor-Papadimitriou et al. 1982) are cultured in FDMEM medium in flasks at 10% CO₂ and 37°C. When confluent, cells are washed with magnesium- and calcium-free phosphate buffered saline (PBS). PBS is decanted and cells detached by adding 5 ml of trypsin (0.05%) in EDTA (0.02%). PBS is added to a volume of 50 ml. The cell pellet obtained by centrifugation (1000 g, 5 min) is re-suspended in 10 ml of FDMEM. Cell counts in the final suspension are determined using a haemocytometer, and the cell density is adjusted to 25000/ml with FDMEM.

For microtiter assays, cells (5000 in 200 µl medium) are inoculated into the inner wells of 96-well plates. The outer edge wells of the plate contain 200 µl of 10% fetal bovine serum in PBS. After 24 h at 37°C, 10% CO₂, plating medium is replaced with FDMEM containing test material or standard in 200 µl. Standard substances are: madecassoside (50 nM–5 mM), asiaticoside (100 nM–10 mM), dissolved in methanol, and dithranol (0.33–170 µM) dissolved on DMSO. Each sample concentration is tested with 6 replicates on each of three separate plates. Cells exposed to FDMEM alone provide 100% growth control. Cells are incubated for 7 days at 37°C, 10% CO₂ prior to carrying out the sulphorhodamine B assay.

For the sulphorhodamine B (SRB) assay (Skehan et al. 1990), cells are fixed by layering 100 µl of ice-cold 50% trichloroacetic acid on top of the growth medium. Cells are incubated at 4°C for 1 h, after which plates are washed 5 times with cold water, excess water drained off and the plates left to dry in air. SRB stain (50 µl; 0.4% in 1% acetic acid) (Sigma) is added to each well and left in contact with the cells for 10 to 30 min after which they are washed with 1% acetic acid, rinsing 4 times until only the dye adhering to the cells is left. The plates are dried and 100 µl of 10 mM Tris buffer added to each well to solubilize the dye. The plates are shaken gently for 5 min on a plate reader

and the absorbance is read at 550 nm using a Titertek Multiscan MCC/340 II plate reader.

EVALUATION

Mean optical density (OD \pm SD) is calculated for each concentration from the six replicate wells in a single plate. The data is used to plot a dose response curve from which IC_{50} values \pm SD are obtained.

MODIFICATIONS OF THE METHOD

Fogh et al. (1993) described an *ex vivo* skin model. Keratinized psoriatic skin samples were incubated in the presence of the calcium ionophore a23187 and arachidonic acid for 45 min at 37°C. After extraction of lipids, eicosanoids were determined by reversed-phase high-performance liquid chromatography in combination with specific radioimmunoassays.

Hager et al. (1999) described long-term culture of murine epidermal keratinocytes.

Hanley et al. (1998) found that the differentiation of keratinocytes in cultures from human foreskin was stimulated by activators of the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPAR α).

Rivier et al. (1998) reported differential expression of PPAR subtypes during the differentiation of human keratinocytes. When normal human keratinocytes were induced to differentiate by shifting the culture medium to a high Ca^{2+} concentration, the expression of PPAR α and PPAR γ mRNA was increased, whereas that of PPAR δ remained unchanged. In lesional compared with unlesional psoriatic epidermis, the expression of PPAR α and PPAR γ was reduced, indicating that these two subtypes are tightly linked in the epidermal differentiation process.

Naik et al. (1999) found that human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene.

Muga et al. (2000) reported that 8S-lipoxygenase products activate PPAR α and induce differentiation in murine keratinocytes. Transgenic mice with 8S-lipoxygenase targeted to keratinocytes were generated through the use of a loricrin promoter. In cultured keratinocytes, PPAR α was identified as a crucial component of keratin-1 induction through transient transfection with expression vectors for PPAR α , PPAR γ , and a dominant negative PPAR as well as through the use of known PPAR agonists.

Ellis et al. (2000) found that ligands for PPAR γ inhibit keratinocyte proliferation. Troglitazone improved

psoriasis and showed normalization in these models of proliferative skin disease.

Kömüves et al. (2000) studied keratinocyte differentiation in hyperproliferative epidermis and found that topical application of PPAR α activators restores tissue homeostasis.

Rosenfield et al. (2000) reviewed PPARs and skin development.

Sakamoto et al. (2000) described activation of human PPAR subtypes by pioglitazone.

Tan et al. (2001) studied the roles of PPAR β/δ in the keratinocyte's response to inflammation. The authors showed by the use of cultures of primary keratinocytes from wild-type and PPAR $\beta/\delta^{-/-}$ mice that inflammatory signals, such as tumor necrosis factor α (TNF α) and interferon β (INF β), induce keratinocyte differentiation. The proinflammatory cytokines initiate the production of endogenous PPAR β/δ ligands, which are essential for PPAR β/δ activation and action.

Thuillier et al. (2002) found inhibition of PPAR-mediated murine keratinocyte differentiation by lipoxygenase inhibitors. The effect was mediated primarily through PPAR α and PPAR γ .

Zhou et al. (2002) performed genetic analysis of four novel PPAR γ splice variants in monkey macrophages.

Hong et al. (2003) described PPAR γ -dependent anti-inflammatory action of rosiglitazone in human monocytes, whereby the suppression of TNF α secretion is not mediated by Lipid phosphatase PTEN regulation.

Kojo et al. (2003) evaluated human PPAR subtype selectivity of a variety of anti-inflammatory drugs based on a novel assay for PPAR δ (β).

Kuenzli and Saurat (2003) reviewed the role of PPARs in cutaneous biology.

Westergaard et al. (2003) studied expression and localization of PPARs and nuclear factor κ B in normal and lesional psoriatic skin.

Gosh et al. (2004) found disruption of transformation growth factor signaling and profibrotic responses in normal skin fibroblasts by PPAR γ .

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- 25 μ g/ml mitomycin C for 30 min at 37°C in an atmosphere of 5% CO₂ and 100% humidity and then washed three times with RPMI-1640 medium. The stimulator cells are pooled at 0.5–1.0 \times 10⁶ cells per ml per donor in RPMI-1640 medium. Cells are cultured in medium consisting of RPMI-1640 supplemented with 4 mM L-glutamine, 500 μ M 2-mercaptoethanol, 10% fetal bovine serum, 25 units/ml penicillin, and 25 μ g/ml streptomycin. Mixed leukocyte reactions are performed in 96 well flat-bottom plates (Corning, Acton MA) in a final volume of 220 μ l, containing 20 μ l of culture medium with or without test compound or standard (ascomycin, purified from a fermentation of *Streptomyces hygroscopicus* ssp. *ascomyceticus* ATCC 14891 and characterized according to Or et al. 1993), 100 μ l of responder cells (1 \times 10⁶ cells per ml) and 100 μ l of stimulator cells (2–4 \times 10⁶ cells per ml). Cultures are incubated at 37°C in an atmosphere of 5% CO₂ and 100% humidity for 4 days. On day 4, 0.5 μ Ci of [³H]thymidine is added to each well during the last 6 h of culture. Cultures are harvested onto glass-fiber mats using a 96 well harvester. [³H]Thymidine uptake is measured by direct β -counting using a Matrix 9600 β -counter (Packard, Meriden, CT).

P.4.2.2

Effect on T Lymphocytes

PURPOSE AND RATIONALE

Psoriasis vulgaris is considered as an inflammatory cell-mediated autoimmune disorder (Uyemura et al. 1993; Gottlieb et al. 1994; Bata-Csörgö et al. 1995; Austin et al. 1999). Lesional skin T cells contribute pro-inflammatory/type 1 (T1) cytokines to initiate and maintain the cell-mediated keratinocyte hyperplasia in inflammatory lesions. Atopic dermatitis is characterized by the presence of Th2 cell and their respective cytokines (Cooper 1994; Leung 1997). A therapeutic agent down-regulating both Th1 and Th2 cytokine production would be desirable (Mollison et al. 1999; Santamaria et al. 1999).

PROCEDURE

Human Mixed Leukocyte Reaction (MLR) Assay

Fifty milliliters of sodium heparinized blood, obtained from normal, unrelated donors, is mixed with an equal volume of Dulbecco's phosphate buffered saline (DPBS). Peripheral blood mononuclear cells (PBMC) are isolated by density centrifugation at 400 g over Histopaque-1077. A sufficient number of PBMC are washed three times in RPMI-1640 medium and used as responder cells. The remaining PBMC (stimulator cells) are washed as above and treated with

Inhibition of IL-2 Production in Human Whole Blood and PBMC

Fifty milliliters of venous blood from normal donors is drawn into tubes containing sodium heparin. Twenty-five milliliters of blood is used directly. PBMC are isolated from the remaining sample as described above and cultured at 1 \times 10⁶ cells per ml with supplemented RPMI-1640. Whole blood or PBMC are induced to secrete IL-2 with 50 ng PMA per ml plus 1 μ g ionomycin per ml. Immunosuppressive potency of test compound vs. standard is determined by measuring the inhibition of IL-2 secretion. Assays are performed in 96 well flat-bottom plates in a volume of 210 μ l, which includes 190 μ l of whole blood or PBMC (1 \times 10⁶ cells per ml), 10 μ l of PMA (1 μ g/ml) and ionomycin (20 μ g/ml) mixture and 10 μ l of serially diluted test compound. Plasma or tissue culture supernatants are collected 24 h later and IL-2 concentrations determined by ELISA.

Porcine MLR Assay

Fifty milliliters of sodium heparinized venous blood is drawn from pigs and diluted with an equal volume of 0.9% saline. PBMC are isolated by density centrifugation for preparation of responder and stimulator cells as described above. MLR are performed as described for human MLR using 100 μ l of responder

cells (2×10^6 cells per ml) and 100 μ l of stimulator cells ($0.5\text{--}4 \times 10^6$ cells per ml).

Rat MLR Assay

Popliteal, inguinal, and mesenteric lymph nodes from newly sacrificed Lewis rats and spleens from Brown Norway rats are aseptically removed. Single cell suspensions of splenocytes and lymphocytes are prepared using forceps and a hemostat to macerate the tissues. Red blood cells in the splenocyte suspension are lysed by 2 min incubation at ambient temperature in red blood cell lysis buffer containing 0.14 M NH_4Cl in 0.0167 M Tris-HCl, pH 7.2. Responder cells from Lewis rat lymph nodes and stimulator cells from Brown Norway rat spleens are washed three times in RPMI-1640 medium and then sedimented at 400 g for 10 min. Stimulator cells are prepared as for human, and the assay is conducted in serum-free AIM-V medium supplemented with 1% Antibiotic-Antimycotic solution and 50 μ M 2-mercaptoethanol, using an optimized cell ratio of 100 μ l of responder cells (2×10^6 cells per ml) and 100 μ l of stimulator cells ($1\text{--}2 \times 10^6$ cells per ml) and an incubation period of 4 days.

Mouse MLR Assay

Spleens are aseptically removed from newly sacrificed $\text{C}_3\text{H}/(\text{C}_3\text{H}/\text{HeNcrIBR})$ and BALB/c mice. Splenocytes are prepared and the assay conducted as for rat, using an optimized cell ratio of 100 μ l of responder cells (4×10^6 cells per ml) and 100 μ l of stimulator cells (1×10^6 cells per ml).

Concanavalin A-Induced Guinea Pig Lymphocyte Proliferation Assay

Spleens are aseptically removed from newly sacrificed guinea pigs. Single cell suspensions of splenocytes are prepared as for rat using 4 min incubation in lysis buffer to remove red blood cells. Splenocytes are washed three times in RPMI-1640 medium and adjusted to 6.25×10^5 cells per ml in culture medium consisting of RPMI-1640 supplemented as described for human MLR. Concanavallin A-induced proliferation reactions are performed in a volume of 200 μ l, containing 20 μ l of culture medium with or without test compound or standard, 160 μ l of guinea pig splenocytes and 20 μ l concanavallin A (20 μ g/ml). Cultures are incubated for 3 days, pulse labeled on the last day with [^3H]thymidine, harvested and counted as described.

Inhibition of Cytokine Secretion

Serially diluted test compound or standard is added to 96 well plates with PBMC in RPMI-1640 medium at

1×10^6 cells per ml. PMA and ionomycin are added to effect 10 ng per ml and 500 ng per ml concentrations, respectively. Supernatants from these cultures are collected 24 h later after centrifugation and stored at -80°C until use. Concentrations of IL-2 and IFN- γ in the supernatants are determined by ELISA. For assessing inhibition of IL-4 and IL-5 secretion in T cells by the test compound, CD4^+ cells are isolated from PBMC using a T cell subset enrichment column (RandD Systems, Minneapolis, MN). Purified CD4^+ cells are cultured with serially diluted test compound and PMA at 10 ng per ml in plates previously coated with 100 ng per ml solution of anti CD3 antibody (Immunotech, Westbrook, ME). Supernatants are collected after centrifugation 40 h later. IL-4 and IL-5 concentrations in the supernatants are determined by ELISA.

EVALUATION

Dose response curves for test compound and standard are established and IC_{50} values for inhibition calculated.

MODIFICATIONS OF THE METHOD

Gillitzer et al. (1996) studied neutrophil migration in psoriatic lesions as a model for neutrophil chemotaxis.

Kunstfeld et al. (1997) investigated the migration of inflammatory T cells from psoriasis through superficial vascular plexus and through deep vascular plexus endothelium. Superficial and deep plexus human skin was placed separately into SCID mice.

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P.4.3

Psoriasis Models in Normal Animals

P.4.3.1

Mouse Tail Model for Psoriasis

PURPOSE AND RATIONALE

The mouse-tail test was introduced by Jarrett and Spearman (1964). The model is based on the induction of orthokeratosis in those parts of the adult mouse-tail, which have normally a parakeratotic differentiation. Quantification of orthokeratosis was achieved by morphometric evaluation of the scales with the aid of a semiautomatic evaluation unit (Bosman et al. 1992; Bosman 1994; Sebök et al. 1996).

PROCEDURE

Male albino NMRI mice weighing 25–27 g are used.

The tails are treated locally with 0.1 ml ointment applied to the proximal part of the tail. For the contact time of 2 h a plastic cylinder is slipped over the tail and fixed with adhesive tape. At the end of contact time the cylinders are removed and the tails washed. Animals are treated once daily, 5 times a week, for 2 weeks. Five to 8 animals are used per dosage group. Two hours after the last treatment the animals are sacrificed and the tails prepared histologically (fixation in 4% formalin, paraplastic embedding). Longitudinal sections of about 5 μm thickness are prepared and stained with hematoxylin-eosin.

The epidermal thickness is measured as the distance between the dermo-epidermal borderline and the be-

ginning of the horny layer. Five measurements per animal are made in each of 10 scales. Out of these 50 measurements the mean for the individual animals is calculated.

The sections are examined for the presence of a granular layer or isolated granular cells induced in the previously parakeratotic skin areas (10 sequential scales per animal). The measurements are carried out at the border of the scale with a semiautomatic image evaluation unit (VIDS III, AI TEKTRON). The distances are obtained in pixels (1 pixel = 1.2120 μm).

Quantitative values of orthokeratosis are obtained by measuring the length of the granular layer per scale (A) and the whole scale length (B). The whole scale length is defined as the length of the scale lying between two adjacent hair follicles, beginning and ending at the turning point between hair follicle and scale. Percent keratosis is calculated by the formula:

$$(A/B) \times 100.$$

EVALUATION

Ten sequential scales per animal are measured and the results given in % orthokeratosis per scale. Five to 8 animals are taken for one drug concentration or control group. Thus 50–80 individual orthokeratosis values are obtained per test group. Mean and standard error of the mean are calculated per animal and per group. From the individual orthokeratosis values per dosage group (50–80 scales) a frequency distribution is constructed. Therefore the values (ranging from 0 to 100% orthokeratosis) are grouped into classes with a constant class interval of 10% (class 1: 0–10%; class 2: 10.1–20%; class 10: 90.1–100% orthokeratosis). The frequency per class is calculated in %:

$$\text{class frequency} = \frac{\text{no. of scales in the class}}{\text{total no. of scales}} \times 100.$$

For every class the cumulative frequency is constructed by adding the frequencies of all foregoing classes.

Due to a non-Gaussian distribution of the orthokeratotic values (100% is the maximal effect), the Mann-Whitney U -test is used.

The efficacy of test compounds on epidermal differentiation is calculated from the mean length of orthokeratosis after treatment with the substrate (Ok_s) and with salicylic acid as control (Ok_c) using the formula:

$$(Ok_s - Ok_c)/(100 - Ok_c) \times 100.$$

MODIFICATIONS OF THE METHOD

Nagano et al. (1990) studied the effect of tumor necrosis factor in the mouse-tail model of psoriasis.

Beyaert et al. (1992) induced a psoriasiform inflammatory reaction in mice by subcutaneous injection of a combination of tumor necrosis factor and lithium chloride.

Sebök et al. (2000) used the mouse tail test to compare tazarotene, a receptor-specific retinoid, with the classical topical antipsoriatic compound dithranol.

Worm et al. (2001) studied the effects of retinoids on *in vitro* and *in vivo* IgE production in ovalbumin-sensitized BALB/c mice.

Several studies were performed in **guinea pigs** (Miller and Ziboh 1990; Kumar et al. 1992; Maini et al. 1999). Tuzun et al. (1993) reported psoriasis lesions in guinea pigs receiving propranolol; however, their results could not be confirmed by Wolf et al. (1994).

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P.4.3.2

Rat Ultraviolet Ray B Photodermatitis Model for Psoriasis

PURPOSE AND RATIONALE

Rat ultraviolet ray B photodermatitis has been proposed as an experimental model of psoriasis vulgaris by Nagakuma et al. (1995).

PROCEDURE

Male Wistar rats weighing around 300 g are used. Hair on the dorsal skin is clipped and carefully shaved. An area (1.5 × 2.5 cm) on one side of the flank is irradiated for 15 min (1.5 J/cm²) at a vertical distance of 20 cm with UV-B lamps. A biphasic erythema is observed. Immediately after irradiation, initial faint erythema appears, disappearing within 30 min. The second phase of erythema starts 6 h after the irradiation and gradually increases, peaking between 24 and 48 h. The color is brownish-red, and the reaction is confined to the exposed area with a sharp boundary. By 48–72 h after irradiation, dark-brown scale is formed on the erythematous lesion. Pieces of the scale are relatively thick. The scale separates and the erythema decreases daily. The skin sites return to normal about 10 days after irradiation.

The irradiated rats are sacrificed after various time intervals by decapitation under ether anesthesia. Skin biopsies are taken immediately, fixed in 10% formalin and embedded in paraffin. Tissue sections (4 μm thick) are stained with hematoxylin and eosin. The numbers of the keratinocyte layers, including the basal layer, are counted by direct microscopy.

For DNA labeling (Morimoto et al. 1991), 20 mg/kg 5-bromo-2-deoxyuridine (BrdU) is administered *i.p.* 8 h before decapitation. Frozen tissue sections (8 μm) are prepared with a cryostat. The sections are then fixed in acetone for 10 min, 4% paraformaldehyde containing 1% CaCl₂ (pH 7.0) for 10 min and 1% glutaraldehyde for 5 min. After fixation, sections are digested with 0.006% pepsin in 10 mM HCl for 10 min at 37°C, and incubated in 4 M HCl for 30 min at ambient temperature to denature DNA. They are then incubated with anti-BrdU mouse monoclonal antibody and then

with alkaline phosphatase-conjugated anti-mouse IgG sheep IgG antibody. Antibody-binding sites are visualized with naphthol and fast red at pH 9 in the dark. The number of labeled as well as unlabeled cells at the epidermal basal layer or outer root sheath cell layers of the hair follicles are counted in five high-power fields to a total of at least 100 cells. The intensity of BrdU incorporation is demonstrated as a labeling index (%) with the following formula:

$$\text{labeling index} = \frac{\text{no. of labelled cells}}{\text{total cell no. counted}} \times 100$$

EVALUATION

Chronological changes of thickness of keratinocytes at the epidermis and the hair follicles as well as of DNA labeling indices are plotted as time-response curves.

MODIFICATIONS OF THE METHOD

Kippenberger et al. (2001) found that activators of peroxisome proliferator-activated receptors (PPARs) protect human skin from ultraviolet-B-light induced inflammation. In an *in vitro* model with human keratinocytes inflammation was mimicked by irradiation with ultraviolet light. Activators of PPAR α were shown to reverse ultraviolet-B-light-mediated expression of inflammatory cytokines.

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P.4.4

Hyperproliferative Epidermis in Hairless Mice

PURPOSE AND RATIONALE

Epidermal differentiation and barrier development *in vivo* are influenced by nuclear hormone receptors (Hanley et al. 1997, 1998, 1999; Kömüves et al. 1998; Rosenfield et al. 2000; Smith et al. 2001a, 2001b; Kuenzli and Saurat 2003a, 2003b). Kömüves et al. (2000) studied the effect of PPAR α activators on keratinocyte differentiation in hyperproliferative epidermis in hairless mice.

PROCEDURE

Subacute epidermal hyperproliferation is induced by repeated barrier abrogation with acetone treatment (Denda et al. 1996). Animals are treated twice a day with acetone until the transepidermal water loss reaches 8–10 mg per cm² per h (measured on four to five different spots) as determined by an electrolyte water analyzer.

Chronic epidermal hyperproliferation due to essential fatty acids deficiency is achieved by feeding an isocaloric diet free of essential fatty acids after weaning for 2–3 months (Man et al. 1993).

To test the effect of PPAR α activators, mice are treated on one flank side with solutions of the drug dissolved in propylene glycol/ethanol (3/7) twice a day for 3 days. After sacrifice, tissues are collected for histology, *in situ* hybridization, or immunohistochemistry.

EVALUATION

Data are presented as mean \pm SEM. Statistical differences are determined using Student's *t*-test.

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P.4.5

Psoriasiform Skin Diseases in Spontaneous Mice Mutations

PURPOSE AND RATIONALE

Nearly 100 mouse mutations have been described as causing some type of abnormality of the skin or hair. These include “asebia”, a mildly hyperkeratotic disorder with sebaceous gland hyperplasia, “ichthyosis”, an example of abnormal hair growth associated with hyperkeratosis, “rhino” and “hairless”, two related examples of congenital follicular malformations, and “flaky skin”, a potential animal model of eruptive psoriasis.

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P.4.5.1

Flaky Skin (*fsn*) Mouse

PURPOSE AND RATIONALE

Flaky skin (*fsn*) is an autosomal recessive mouse mutation that causes pathologic changes in the skin, yielding a papulosquamous disease resembling human psoriasis (Sundberg et al. 1993). In addition, this mutation, assigned to distal chromosome 17, causes anemia and gastric forestomach hyperplasia (Beamer et al. 1995).

The *fsn* mutation arose spontaneously in the A/J inbred strain at The Jackson Laboratory (Beamer et al. 1986). Psoriasiform skin lesions are first evident as focal epidermal hyperplasia and inflammation at 2 weeks of age. These lesions become confluent and diffuse by 3–4 weeks of age and are associated with marked dermal infiltration of lymphocytes and small numbers of neutrophils and macrophages. Mast cell numbers increase significantly in the dermis from 2 weeks of age onward. Diffuse dermal neovascularization accompanies these cutaneous changes (Sundberg et al. 1997). Systemic lesions include progressive and massive papillomatosis of the stratified squamous epithelium of the forestomach, hyperplasia and dysplasia of the glandular stomach, increased apoptosis of cecal entero-

cytes, renal glomerulopathy associated with inflammatory cell infiltrates and fibrosis around the portal triads in the liver, splenomegaly due to massive erythropoiesis, and granulomatous lymphadenitis.

Scanning microscope examination (Morita et al. 1995) reveals a greatly thickened epidermis, and sparsity of hairs and scale accumulations in the epidermal surface. Hair shafts have conspicuous pits, striations, and exophytic protrusions. Nails are bent at a 90-degree angle with surface irregularities and accumulation of scale at the nail base. Transmission electron microscopic examination shows increased epidermal thickness, mitochondrial aberrations, and intraepidermal invasion by neutrophils. Keratin abnormalities are detected using immunocytochemical staining for profilaggrin. At the dermal-epidermal junction, numerous macrophages and mast cells are seen in close proximity to focal dissolutions of the basement membrane. A high density of collagen fibers and cellular infiltrates are evident in the papillary dermis.

Besides lymphadenopathy and mast cell accumulation, elevated serum IgE levels (>7000 fold increase compared with normal littermates), autoimmunity (evidenced by glomerulonephritis with immune complex deposition in the kidneys), are observed in flaky skin mutant mice (Pelsue et al. 1998).

Peripheral lymph nodes of adult mutant (*fsn/fsn*) mice were found to contain almost 10-fold more leukocytes than peripheral lymph nodes from phenotypically normal littermates. Analysis of peripheral lymph node cells using mAbs and flow cytometry revealed that this predominantly lymphoid hyperplasia is characterized by approximately equivalent increases of CD3⁺ T cells and C19⁺ B cells (Abernethy et al. 2000a). A dysregulated expression of CD69 and IL-2 receptor alpha and beta chains was found on CD8⁺ T lymphocytes (Abernethy et al. 2000b). Expression and function of IL-1 beta is increased in psoriasiform skin lesions of flaky skin (*fsn/fsn*) mice (Schön et al. 2001).

However, neutrophils also played a critical role of for the generation of psoriasiform skin lesions in flaky skin mice (Schön et al. 2000). Intraperitoneal injection of the neutrophil-depleting RB6–85C monoclonal antibody resulted in a dramatic reduction of the epidermal thickness.

Similar to active human psoriatic lesions, an increase of epidermal growth factor receptors was observed in *fsn/fsn* mice (Nanney et al. 1996).

Sundberg et al. (1994a) transplanted full-thickness skin grafts from flaky skin mice on the dorsal skin

of genetically athymic nude (*nu/nu*) mice. The grafts maintained the psoriasiform phenotype of the donors.

Backcrosses to different mouse strains suggested several modifier genes affecting the *fsn* phenotype (Sundberg et al. 1994b). As cyclosporine A, in contrast to glucocorticoids, was not effective when used for topical or systemic treatment of *fsn* lesions, it seems that there is no immunologic basis for these lesions. Therefore, it remains uncertain whether the flaky skin mouse can be used to test potential therapeutic compounds.

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P.4.5.2

Asebia (*ab/ab*) Mouse

PURPOSE AND RATIONALE

The asebia mouse was first described by Gates and Kasarek (1965) as hereditary absence of sebaceous glands. The asebia mouse represents a spontaneous mutation in BALB/c mice leading to hyperplasia of the epidermis and chronic inflammatory dermal changes, including enhanced cellularity, edema and elevated mast cell numbers (Josefowicz and Hardy 1978; Brown and Hardy 1988). The circadian rhythms in cell proliferation are suppressed in the chronically hyperproliferative epidermis of the asebia mouse (Brown et al. 1988a). UVB radiation as well as anthralin and tar with UVB further stimulate proliferation in the already hyperproliferative epidermis of the asebia mouse (Brown et al. 1988b, 1989). The gene for the enzyme stearyl-CoA desaturase 1, which is expressed in sebaceous glands, is disrupted in the asebia mouse (Parimoo et al. 1999; Zheng et al. 1999; Miyazaki et al. 2000). Besides *Sdc1* and *Sdc2*, Zheng et al. (2001) identified *Scd3* – a novel gene of the stearyl-CoA desaturase family with restricted expression in the skin. Since T cell and neutrophil infiltrates are not observed in asebia mice, a pathogenesis distinct from psoriasis has been suggested (Schön 1999).

Asebia-2J (*Scd1(ab2J)*): a new allele and a model for scarring alopecia was described by Sundberg et al. (2000).

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P.4.6

Psoriasiform Skin Diseases in Genetically Modified Animals

PURPOSE AND RATIONALE

Based on expression in inflammatory skin disorders, several factors, such as cytokines, are considered to play a crucial role in the pathogenesis of psoriasis and other skin diseases. Transgenic animals are tools to analyze these factors (Meyer 1990; Rothnagel et al. 1990; Sellheyer 1996; Rosenberg et al. 1999).

Bullard et al. (1996) described a polygenic mouse model of psoriasiform skin disease in **CD18-deficient mice**. CD18 deficiency in patients results in recurrent microbial infections, leukocytosis, impaired wound healing, failure of granulocyte emigration, and lack of pus formation (Anderson et al. 1995). A hypomorphic mutation for CD18 was introduced by Wilson et al. (1993) into mice with homozygotes displaying mild leukocytosis, an impaired response to chemically induced peritonitis, and delays in transplantation rejection. When this CD18 mutation was crossed back onto PL/J strain of mice, the development of an inflammatory skin disorder was observed (Bullard et al. 1996). The disease is characterized by erythema, hair loss, and the development of crusts. The histopathology reveals hyperplasia of the epidermis, subcorneal microabscesses, orthohyperkeratosis, parakeratosis, and lymphocyte exocytosis similar to human psoriasis and other hyperproliferative skin disorders. The dermatitis rapidly resolved after subcutaneous administration of dexamethasone.

Dermal infiltrates of macrophages/monocytes within the dermis of clinically uninvolved skin were seen in transgenic mice with epidermal overexpression of K14/IL-1 α , suggesting a role of IL-1 α

for macrophage attraction (Groves et al. 1995). In severely affected animals, inflammation reactions occur that are characterized by a mixed inflammatory infiltrate, and by acanthosis and parakeratosis. **IL-1 α transgenic mice** support a primary role of IL-1 α as an inducer of cutaneous inflammation, which may be helpful for clarifying pathogenesis of psoriasis.

Wilson et al. (1990) reported that expression of the **BNLF-1 oncogene** of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6.

Cook et al. (1997, 1999) found that overexpression of the heparin-binding EGF-related ligand amphiregulin in the epidermis of transgenic mice induces a psoriasis-like cutaneous phenotype. **Transgenic mice with a K14 enhancer/promoter-driven amphiregulin gene** targeted to the epidermis displayed a macroscopic phenotype that included extensive areas of scaling and erythematous skin with marked alopecia. Histological examination revealed hyperkeratosis, focal parakeratosis, acanthosis, mixed leukocytic infiltration that included both CD3-positive T cells in the dermis and epidermis, and a tortuous vasculature.

Two completely opposite phenotypes were observed in **mice expressing K10/BMP-6** (bone morphogenetic protein-6, a member of the TGF- β superfamily) within the epidermis (Blessing et al. 1996). Whereas keratinocyte proliferation was severely reduced in animals with strong and homogenous expression of the transgene, weaker and patchy expression led to marked hyperproliferation.

Rodriguez-Villanueva et al. (1998) reported that **human keratin-1.bcl-2 mice** aberrantly express keratin 6, exhibit reduced sensitivity to keratinocyte cell death induction, and are sensible to skin tumor formation.

The concept of vascular endothelial growth factor (VEGF) being an important angiogenic factor in psoriatic skin (Detmar et al. 1994) was supported by **transgenic mice with constitutive epidermal K14/VEGF expression**. These animals exhibited dilated and constricted dermal microvessels (Detmar et al. 1998). There was also an increased number of dermal mast cells, and leukocyte adhesion and extravasation was enhanced in VEGF transgenic mice. The transgenic delivery of VEGF to mouse skin results in a profound inflammatory condition with many of the cellular and molecular features of psoriasis, including the characteristic vascular changes, epidermal alterations, and inflammatory infiltrates. These symptoms can be effectively reversed by the addition of VEGF Trap, a potent VEGF antagonist (Xia et al. 2003). VEGF-A also promoted lym-

phatic vessel proliferation and enlargement. Combined systemic treatment with blocking antibodies against VEGF receptor-1 (VEGFR-1) and VEGFR-2 potently inhibited inflammation and also decreased lymphatic vessel size (Kunstfeld et al. 2004).

Klement et al. (1996) found that **I κ B α deficiency** results in a sustained NF- κ B response and widespread dermatitis in mice.

Seitz et al. (1998) reported that alterations on NF- κ B function in transgenic epithelial tissue demonstrate a growth inhibitory role for **NF- κ B**.

To determine the role of κ B- α deficient immunocytes in the pathogenesis of skin disease in adult mice, Chen et al. (2000) utilized the RAG2-deficient blastocyst complementation system to generate **RAG2-/-, I κ B- α -/- chimeras**. These animals display a psoriasiform dermatitis characterized by hyperplastic epidermal keratinocytes and dermal infiltration of immunocytes, including lymphocytes. Skin grafts transferred from diseased chimeras to recipient nude mice produce hyperproliferative epidermal keratinocytes in response to stimulation.

Transgenic mice overexpressing suprabasal integrins α_2 , α_5 , and β_1 demonstrate a phenotype similar to psoriasis with cycles of flaking and inflamed skin, suggesting that disrupted keratinocyte integrin-ligand interactions may play a role in hyperproliferative states (Carroll et al. 1995).

Robles et al. (1996) found that **expression of cyclin D1 in epithelial tissue** of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia.

Carroll et al. (1997) used the **involucrin promoter to overexpress** IFN γ in the suprabasal layers of transgenic mouse epidermis. The mice exhibited striking hypopigmentation of the hair due to reduction of DOPA-positive melanocytes. Severely affected mice had reddened skin, growth retardation, hair loss, and flaky skin lesions. The skin was characterized by a dermal infiltrate of T lymphocytes and macrophages/monocytes.

When **scid/scid mice were reconstituted with MHC-matched, but minor histocompatibility mismatched CD4⁺/CD45RB^{hi}T lymphocytes**, almost all of the animals developed skin lesions similar to human psoriasis within 4–8 weeks after transfer (Schön et al. 1997). The psoriasiform skin lesions did not develop in recipients of unfractionated splenocytes or CD4⁺/CD45RB^{lo} T cells, indicating that T cell dysregulation is the primary pathogenic factor in this model. When recipients of CD4⁺/CD45RB^{hi} T cells were treated with either cyclosporine A or UVB ir-

radiation, the psoriasiform lesions were dramatically improved demonstrating that immunosuppressive therapies are efficacious. The model was proposed as a T cell initiated murine model of inflammatory skin lesions (Schön and Parker 1997).

Mann et al. (1993) found that mice with a **null mutation of the TGF- α gene** have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation.

Turksen et al. (1992) described psoriasiform changes in the skin of transgenic mice with **overexpression of interleukin 6**.

Guo et al. (1993) reported that targeting expression of **keratinocyte growth factor** to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice.

Hong et al. (1999) showed that IL-12, independently of IFN-gamma, plays a crucial role in the pathogenesis of a murine psoriasis-like disorder. Only a few *scid/scid* mice develop skin lesions when CD4⁺/CD45RB^{hi} are transferred alone. Coadministration of LPS plus IL-12 or staphylococcal enterotoxin B in *scid/scid* mice one day after **CD4⁺/CD45RB^{hi} cell transfer** greatly enhances disease penetrance and severity.

Schön et al. (2000) reported a cutaneous inflammatory disorder in **integrin alpha E (CD103)-deficient mice**. Skin inflammation correlated with alpha E deficiency in mice with a mixed 129/Sv \times BALB/c background.

Transgenic rats expressing human HLA-B27 and β_2 -microglobulin develop psoriasiform skin changes as part of a multi-organ inflammatory disease (Hammer et al. 1990). In the most severely affected lines, psoriasiform lesions first occurred at about 20 weeks of life and were observed in 10% to 80% of animals. Male appeared to be more prone to skin changes than females (Taurog et al. 1993, 1994, 1999). Similar to human pathogenesis, T cells seem to be the most important factor in HLA-B27 transgenic rats (Breban et al. 1996).

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P.4.7

Xenotransplantation of Human Psoriatic Skin

PURPOSE AND RATIONALE

Xenotransplantation models for psoriasis involve the transfer of human psoriatic skin to animals. First stud-

ies were performed by transplantation of involved psoriatic and non-psoriatic human skin onto congenitally athymic (nude) mice (Krueger et al. 1975, 1985; Baker et al. 1992). More recently, human psoriatic skin grafts were transplanted onto SCID mice (Nickoloff et al. 1995; Boehncke et al. 1994, 1997, 1999; Gilhar et al. 1997; Sugai et al. 1998; Dam et al. 1999; Raychaudhuri et al. 2001) allowing screening of anti-psoriatic drugs.

PROCEDURE

Keratome biopsies (7 × 2 × 0.05 cm containing both dermis and epidermis) are obtained from clinically symptomless skin of patients with psoriasis or from psoriatic plaques. Prior to the procedure, the skin is defined and infiltrated with 1% lidocaine and epinephrine 1:200. The full-thickness skin biopsy is dissected into 12 grafts. CB-17 SCID mice at an age of 6–8 weeks are anesthetized with an i.p. injection of 1.56 mg phenobarbital before transplantation of the human xenografts on the flank area. The grafts are sutured with absorbable 6–0 Vicryl Rapid suture and covered with Xeroform dressings for one week. Animals transplanted with the psoriatic plaque are then randomized into groups receiving PBS as negative controls or 0.15 mg cyclosporine A or the test compound intradermally into the xenografts. Within 4 weeks, the animals are sacrificed by CO₂ asphyxiation and 4 mm punch biopsies are obtained from each xenograft. Biopsies are fixed in 10% neutral-buffered formalin for paraffin embedding.

EVALUATION

A semiquantitative scale is used to indicate the extent of new vessel formation (angiogenesis) that can be seen between the xenograft and the underlying fascia muscularis. A calibrated eyepiece microscope is used for estimating the epidermal thickness in the vertical sections. Statistical differences are calculated using Student's *t*-test for multiple comparisons.

MODIFICATIONS OF THE METHOD

Using this model, Raychaudhuri et al. (2004) found that a high-affinity nerve growth factor receptor blocker improves psoriasis.

Nickoloff et al. (1995) studied severe combined immunodeficiency mouse and human psoriatic skin chimeras. Autologous blood-derived CD4⁺ cells injected into symptomless transplanted psoriatic skin engrafted onto SCID mice produced full-fledged psoriatic lesions (Wrone-Smith and Nickoloff 1996; Sugai

et al. 1998; Nickoloff and Wrone-Smith 1999; Nickoloff et al. 1999; Nickoloff 2000).

Yamamoto et al. (1998) studied the effects of superantigen-driven peripheral blood mononuclear cells on the persistence of psoriasiform epidermis and on the cytokine gene expression of grafted psoriatic skin. Staphylococcal enterotoxin B-stimulated peripheral blood mononuclear cells from psoriatic patients were repeatedly injected under the grafted full-thickness involved psoriatic skin onto severe combined immunodeficient mice. A persistence of the psoriasiform epidermis was found after 5 weeks. E-selectin expression was observed on endothelial cells in the upper epidermis of the mice.

Tissue specificity of E- and P-selectin ligands in Th1-mediated chronic inflammation was studied by Chu et al. (1999).

Zeigler et al. (2001) used the human psoriatic skin-SCID mouse transplant model to compare the effect of antibody to CD11a with ciclosporin and clobetasol propionate.

Zollner et al. (2002) used a SCID-hu model and found that proteasome inhibition reduces superantigen-mediated T cell activation and the severity of psoriasis.

Boyman et al. (2004) presented an animal model in which skin lesions developed spontaneously when symptomless prepsoriatic human skin was engrafted onto AGR129 mice, deficient in type I and type II interferon receptors, and for the recombination activating gene 2. Upon engraftment, resident human T cells in prepsoriatic skin underwent local proliferation.

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P.5

Scleroderma Models

P.5.1

Scleroderma Models in Chicken

Scleroderma is an autoimmune disorder which occurs in the severe systemic form, a localized scleroderma (morphoea) and as lichen sclerosus et atrophicus. Chickens of the University of California line 200 (**UCD-200 chickens**) developed an inherited inflammatory fibrotic disease, closely resembling human progressive systemic sclerosis (scleroderma) (Gershwin et al. 1981; Van de Water et al. 1984; Boyd et al. 1991; Gruschwitz et al. 1991, 1993; Herold et al. 1992; Needleman 1992; Brezinscheck et al. 1993; Ausserlechner et al. 1997; Nguyen et al. 2000). An acute inflammatory stage started at an age of about 60 days after hatching, leading to fibrosis with fast progression with severe lymphocytic infiltration and excessive accumulation of collagen in skin and internal organs. A sequential increase of type VI, type I and type II procollagen transcripts and a progressive increase of autoantibodies to histone, to ssDNA and to dsDNA was found.

A further strain of chickens with progressive systemic sclerosis (UCD 206) has been identified (Duncan et al. 1995; Sgonc et al. 1995).

Warda et al. (2003) analyzed the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay.

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P.5.2

Scleroderma Models in Mice

Jimenez and Christner (2002) gave a survey on murine animal models of systemic sclerosis.

The **tight-skin mouse** is regarded as an experimental model for scleroderma (Walker et al. 1990; Muryoi et al. 1992; Delany and Brinkerhoff 1993; Kasturi et al. 1993, 1994; Wallace et al. 1994; Pablos et al. 1995; Frondoza et al. 1996). This mutant mouse develops autoantigens specific for scleroderma target tissues. The tight-skin disease was first discovered in 1967 at the Jackson Laboratory in the B10.D2(58N)/Sn mouse strain. The disease occurred spontaneously. Green et al. (1976) have identified the *tsk* locus and shown it to be linked to pallid locus. The tight skin character is transmitted as an autosomal dominant trait and homozygous *tsk/tsk* mice die *in utero*. The striking feature of the disease is the presence of thickened skin, firmly bound to subcutaneous and deep muscular tissue, with excessive accumulation of collagen in skin and internal organs (Menton et al. 1978; Osborn et al. 1983). Synthesis and accumulation of type I collagen is markedly increased (Jimenez et al. 1986; Ong et al. 1998). Biochemical studies have shown that the prolyl hydroxylase and glycosaminoglycan content of the affected tissue is increased (Ross et al. 1987).

Dermal fibrosis in the tight-skin mouse was reduced after local application of halofuginone, an inhibitor of collagen type I synthesis (Pines et al. 2001).

Additionally, the **tight skin 2 mouse** has been described as an animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration (Christner et al. 1995, 1996; Wooley et al. 1998; Sgonc et al. 1999).

Bleomycin-induced scleroderma in genetically mast cell deficient WBB6F1-W/W^V mice was described as an animal model of sclerotic skin (Yamamoto et al. 1999a–d, 2000; Yamamoto and Nishioka 2001, 2002; Yamamoto 2002).

Murine sclerodermatous graft-versus-host disease is considered as a model for human scleroderma (Claman 1990; Schiltz et al. 1994; McCormick et al. 1999; Zhang et al. 2002, 2003; Atamas and White 2003). Fibrosis in sclerodermatous graft-versus-host disease is driven by infiltrating TGF- β ₁-producing mononuclear cells. Zhang et al. (2002) characterized the origin and types of cutaneous effector cells, the cytokine and chemokine environments, and the effects of anti-TGF- β antibodies on skin fibrosis, immune cell activation markers, and collagen and cytokine synthesis.

Dong et al. (2002) transplanted skin from scleroderma patients to SCID mice and investigated the skin xenografts for factors downstream of TGF- β . Deficient expression of *Smad7* was found as a putative molecular defect in scleroderma.

A syndrome resembling human systemic sclerosis (scleroderma) in **MRL/lpr mice lacking interferon-receptor** was described by Le Hir et al. (1999).

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P.6 Pemphigus Models

P.6.1 Experimentally Induced Pemphigus in Mice

PURPOSE AND RATIONALE

Pemphigus is defined as a group of spontaneously occurring disorders of skin and mucosae in which blisters and erosions form within the epidermis due to loss of intercellular contact (Marks 1987). Pemphigus occurs also spontaneously in domestic animals, such as dogs, cats, and horses (Hurvitz 1980; Scott et al. 1983; Sueki et al. 1997). Various attempts were made to study experimentally induced pemphigus in mice (Buschard et al. 1981; Takahashi et al. 1985; Anhalt et al. 1986a, b; Rock et al. 1990; Juhasz et al. 1993; Allbritton et al. 1997; Koch et al. 1997; Fan et al. 1999;

Mahoney et al. 1999; Amagai et al. 2000; Zillikens et al. 2001, Rädisch et al. 2002; Hashimoto 2003).

Fan et al. (1999) screened four strains of female mice (BALB/c, DBA/1, SJL/J, and HRS/J) for their ability to produce pathogenic anti-desmoglein 3 antibodies. Only BLB/c mice immunized with full length desmoglein 3 can produce pathogenic antibodies capable of causing acantholysis in human foreskin in culture and blistering in neonatal mice.

PROCEDURE

Six- to 8-week-old female BALB/c, DBA/1, SJL/J, and HRS/J are immunized four times with 20 µg/mouse of purified desmoglein 3 protein in CFA (on days 1, 10, 20, and 30), four times with 20 µg/mouse of extracellular domain of desmoglein 3 (on days 40, 50, 60, and 70), and twice with 20 µg/mouse of re-folded desmoglein 3 (on days 80 and 90), then they are boosted twice more with 20 µg/mouse of extracellular domain of desmoglein 3 in IFA by i.p. inoculation (on days 97 and 104). Control groups of mice are similarly immunized with BSA.

IGs from 10 ml of pooled serum from mice immunized with desmoglein 3 are precipitated with 40% ammonium sulfate, dialyzed against PBS twice, lyophilized, and reconstituted in water to 1 ml. One hundred µl of the reconstituted antibodies/mouse is injected into neonatal BALB/c mice.

EVALUATION

Mice are examined 18–24 h post injection for blister formation. Cross sections containing the blister and comparable areas in control animals are biopsied and frozen sections are prepared for routine histological examination.

MODIFICATIONS OF THE METHOD

Amagai et al. (2000) and Ohshima et al. (2002) developed an active autoimmune disease model for pemphigus using autoantigen knockout mice.

Xu et al. (2001) characterized BALB/c mice B lymphocyte autoimmune responses to skin basement membrane component type XVII collagen, the target antigen of the autoimmune skin disease bullous pemphigoid. The mice were immunized with peptides of the human and/or the murine-equivalent BPAg2 pathogenic NC16A domain.

Using keratinocytes from plakoglobin knockout mice, Caldelari et al. (2001) studied the role of the Armadillo protein plakoglobin in pemphigus vulgaris.

Nguyen et al. (2004) reported that pemphigus vulgaris IgG and methylprednisolone exhibit reciprocal

effects on keratinocytes. The therapeutic effects of methylprednisolone may be due to both the upregulated synthesis and the post-translational modifications of keratinocyte adhesion molecules.

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P.7 Ichthyosis Vulgaris Models

P.7.1

Experimentally Induced Ichthyosis in Mice

PURPOSE AND RATIONALE

Ichthyosis vulgaris is a heterogeneous autosomal skin disease characterized by dry, scaly skin, mild hyperkeratosis, and a decreased or absent granular layer that either lacks, or contains morphologically abnormal, keratohyalin granula (Anton-Lamprecht and Hofbauer 1972). Both the skin of ichthyosis vulgaris patients and keratinocytes cultured from affected individuals exhibit reduced or absent profilaggrin mRNA and protein levels (Sybert et al. 1985). The symptoms and the genetics of the ichthyotic (*ic/ic*) mouse were described by Spearman (1960), Green et al. (1974), Jensen and Esterly (1977), Holbrook (1989). Presland et al. (2000) demonstrated loss of normal profilaggrin and filaggrin in flaky tail (*ft/ft*) mice and proposed this as an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris.

Elias et al. (1983) and Chung et al. (1984) induced ichthyosis in the hairless mouse by treatment with diazacholesterol and used this as an assay for comparative potency of topical retinoids.

PROCEDURE

Male hairless mice, 2–3 months old, are fed either a normal laboratory diet supplemented with 60 mg/kg/day of 20,25-diazacholesterol or normal laboratory diet. Diazacholesterol blocks the conversion of Δ^{24} -reduction of desmosterol to cholesterol (Anderson and Martt 1965), and as a result desmosterol accumulates in stratum corneum lipids rather than cholesterol. Ichthyotic changes generally become apparent after 8–12 weeks and are most pronounced over the back and tail. With the exception of some reduction in body weight in comparison with controls, the animals appear healthy. Since the tail manifests the most exaggerated scaling, this site is used for topical drug applications. As the animals become ichthyotic, the daily dose of diazacholesterol can be lowered to 30 mg/kg and maintained at that level.

The test substances (retinoids) are first solubilized in a small volume of dimethyl sulfoxide and then dissolved in Cremophore EL. A volume of $\sim 100\mu\text{l}$ of each test substance is applied once daily to circumscribed areas of the tail. Treatment groups consist of 3 animals each, and each animal serves as its own control. The drop is first placed on an investigator's gloved index fingers and then spread evenly around a designated band of the tail. Each animal is treated with two concentrations of the test drug. The most proximal and most distal portions of the tail are left untreated as control regions. Applications are continued for 2 weeks. At three- or four-day intervals, and at the termination of the experiment, the clinical response is graded from 0 to 4+, with 0 indicating no response and 4+ indicating removal of all visible scale, leaving a glistening surface.

Prior to biopsy, the skin surface is coated with a thin film of flexible collodion to prevent fragmentation during frozen sectioning. Perpendicular sections of biopsy samples are stained with aqueous 8-anilino-2-naphthalene sulfonic acid, which on fluorescence microscopy depict selectively stratum corneum hydrophobic membrane domains.

EVALUATION

Both control and drug-treated sections are measured in a double-blind manner. The mean and SE from a minimum of five separate regions are tabulated. Significant differences are determined by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Harlequin ichthyosis (ichq): a juvenile lethal mouse mutation with ichthyosiform dermatitis was described by Sundberg et al. (1997).

Shultz et al. (2003) described mutations at the mouse ichthyosis locus within the lamin B receptor gene as a single gene model for human Pelger-Huet anomaly.

Knox and Lister-Rosenoer (1998) described an infantile ichthyosis in **rats** and proposed this as a new model of hyperkeratotic skin disease.

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P.8 Xeroderma Models

P.8.1**Experimentally Induced Xeroderma in Mice****PURPOSE AND RATIONALE**

Xeroderma pigmentosum is an autosomal recessive disorder characterized by hyperphotosensitivity and multiple cancers in association with abnormal DNA repair (Robbins et al. 1974; Satokata et al. 1992). Xeroderma pigmentosum group A (XPA) gene-deficient mice cannot repair UV-induced DNA damage and easily develop skin cancers by UV-irradiation (Nakane et al. 1995; Miyauchi-Hashimoto et al. 1996, 1999). Kuwamoto et al. (2000) tested the involvement of enhanced prostaglandin E₂ production in the photosensitivity in Xeroderma pigmentosum group A model mice and the influence of a prostaglandin synthesis inhibitor.

PROCEDURE

XPA gene-deficient homogeneous mice, age 8–12 weeks, are used (Nakane et al. 1995). The UVB source consists of a bank of fluorescent sunlamps with an emission spectrum from 270 to 375 nm, peaking at 305 nm. The mice are anesthetized by i.p. injection of phenobarbital to keep them immobile during exposure. The ears are irradiated with 250 mJ per cm² of UVB. Immediately after irradiation, the test drug (20 μl of 1% solution of the prostaglandin synthesis inhibitor indomethacin) is applied to the ears. Ear thickness is measured with a dial thickness gauge immediately before irradiation and 1–4 days after irradiation. The amounts of PGD₂, PGE₂, and PGF_{2a} in mouse ears at 0, 24, 48, and 72 after UVB irradiation are determined by enzyme immunoassay.

EVALUATION

Student's *t*-test is employed to determine the statistical difference between means.

MODIFICATIONS OF THE METHOD

De Boer et al. (1999) described a mouse model for the DNA repair/basal transcription disorder trichothiodystrophy, revealing cancer predisposition.

Sun et al. (2003) described a genetic mouse model carrying the nonfunctional xeroderma pigmentosum group G gene. A disrupted XPG allele was generated by insertion of neo cassette sequences into exon 3 of the XPG gene by using embryonic stem cell techniques.

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P.9 Vitiligo Models

P.9.1 Vitiligo in Mice

PURPOSE AND RATIONALE

A **mouse model for vitiligo** was described by Lerner et al. (1986), designated C57BL/6J-vit/vit. The vitiligo mouse has congenital dorsal and ventral white spots as well as progressive replacement of pigmented hairs with each spontaneous molt or after plucking.

Sidman et al. (1996) described pigment epithelial and retinal phenotypes in the vitiligo mivit, mutant mouse.

Tang et al. (1997) studied abnormalities of the electroretinogram in relation to histopathological findings in vitiligo mutant mice.

Iwamoto et al. (1991) established a mouse model for melanoma in which metallothionein/ret transgenic mice express the ret oncogene fused to the metallothionein promoter. Lengagne et al. (2004) reported the occurrence of spontaneous vitiligo in this animal model for human melanoma.

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P.9.2 Smyth Line Chickens

PURPOSE AND RATIONALE

Smyth et al. (1981) developed a mutant line of chickens (DAM line) that is characterized by a high incidence of spontaneous, postnatal, cutaneous amelanosis as a result of an autoimmune phenomenon (Smyth 1989). An intrinsic melanocyte defect, characterized by melanosomes with abnormal irregular surfaces, predisposes these chickens to the pigment disorder (Boissy et al. 1986). Both B cell and T cell compartments of the immune system are involved in the pathology of the disease (Lamont et al. 1982; Erf et al. 1995). Melanocyte-specific antibodies have been detected in the serum of Smyth line chickens (Austin et al. 1992). These autoantibodies cross-react with mouse and human melanocytes and are able to bind pigment within tissues (Searle et al. 1993). Mammalian tyrosinase-related protein-1 is recognized by autoantibodies from vitiligious Smyth chickens (Austin and Boissy 1995). The Smyth chicken model for spontaneous autoimmune vitiligo shows various incidences and degrees of alopecia ranging from alopecia areata-like to universalis-like integumental changes (Smyth and McNeil 1999). Sreekumar et al. (2000) analyzed

the effect of endogenous viral genes in the Smyth line chicken model for autoimmune vitiligo. Erf et al. (2001) reported a strong connection between herpes virus infection and the expression of autoimmune vitiligo in Smyth line chickens. Sreekumar et al. (2001) performed molecular characterization of the Smyth chicken sublines and their parental controls by restriction fragment length analysis and DNA fingerprint analysis. Wang and Erf (2003, 2004) investigated the melanocyte-specific cell-mediated immune response in vitiliginous Smyth line chickens and studied apoptosis in feathers of Smyth line chickens with autoimmune vitiligo.

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P.10

Erythropoietic Protoporphyrin

P.10.1

General Considerations

PURPOSE AND RATIONALE

Erythropoietic protoporphyria (EPP) is an inherited disease that is associated with deficiency of the last enzyme of heme biosynthesis, ferrochelatase, which catalyzes the insertion of ferrous iron into protoporphyrin. Ferrochelatase deficiency is accompanied by photosensitivity syndrome (Bottomley et al. 1975; Cox 1997; Lim and Cohen 1999). It is characteristically manifested by a severe burning pain associated with edema and erythema within a few minutes of exposure to visible light (Cox 1997). Some of these symptoms are related to the extravasation of intravascular contents, such as blood plasma, caused by pronounced damage to endothelial cells (Brun and Sandberg 1991). The defect in ferrochelatase results in accumulation of protoporphyrin (Bottomley et al. 1975; Cox 1997; Lim and Cohen 1999), which may be produced in immature red blood cells, released into the plasma, and accumulate in the cell membranes of endothelial cells due to its hydrophobicity (Brun and Sandberg 1991).

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P.10.2

Animal Models for Erythropoietic Protoporphyrin

PURPOSE AND RATIONALE

Many animal models on erythropoietic protoporphyria are published. Berenson et al. (1992) described a new model of protoporphyric hepatopathy using protoporphyrin overload in unrestrained rats.

Most studies have been performed using mice (Gschnait et al. 1975; Konrad et al. 1975; Cantoni et al. 1983; Tanaka et al. 1993; Libbrecht et al. 2003; Bellingham et al. 1995) described experimental murine protoporphyria induced by griseofulvin. Smith et al. (1997) induced protoporphyria by the orally active iron chelator 1,2-diethyl-3-hydroxypyridin-4-one in C57BL/10ScSn mice.

Many authors (Boulechfar et al. 1993; De Verneul et al. 1995; Meerman et al. 1999; Fontanellas et al. 2001; Abitbol et al. 2005; Han et al. 2005; Navarro et al. 2005) have used genetically altered mice with a recessive inherited ferrochelatase deficiency, originally described by Tutois et al. (1991). Magness et al. (1998) analyzed the human ferrochelatase promoter in transgenic mice.

Pawliuk et al. (2005) reported prevention of murine erythropoietic protoporphyria-associated skin photosensitivity and liver disease by dermal and hepatic ferrochelatase.

Takeshita et al. (2004) performed an L-band electron spin resonance (ESR) study to investigate *in vivo* oxygen radical generation in the skin of the protoporphyria mouse model with visible light exposure.

PROCEDURE

Chemiluminescence techniques have been used to detect $^1\text{O}_2$ and O_2^{*-} using the emission of $^1\text{O}_2$ itself or a chemiluminescent probe (Khan 1981; Nakano et al. 1986). As the luminescence arising from reactive oxygen species is usually very weak compared with the irradiating light, it is technically hard to evaluate reactive oxygen species generation during photodynamic reactions using this method. *In vivo* generation of reactive oxygen species was detected in the skin in erythropoietic protoporphyria disease during irradiation with light, using a technique other than chemiluminescence.

In vivo electron spin resonance (ESR) spectroscopy was used operating at low microwave frequencies as an alternative to chemiluminescence techniques. This technique has enabled the non-invasive measurement of durable free radicals, including a nitroxyl radical, in living experimental animals (Subczynski et al. 1986; Bacic et al. 1989; Ishida et al. 1989; Ferrari et al. 1990; Utsumi et al. 1993). The nitroxyl radical loses its ESR signal on reaction with transient free radicals, such as $^*\text{OH}$ and O_2^{*-} (Takeshita et al. 2004). Free radical reactions were evaluated in various animal models, including hyperoxia (Miura et al. 1992), ischemia–reperfusion (Utsumi et al. 1993), X-ray irradiation (Miura et al. 1997), and streptozotocin-induced diabetes (Sano et al. 1998) using L-band (1–1.2 GHz) and 300 MHz ESR spectrometers with a nitroxyl radical probe (a spin probe). In these experiments, the disease model animal was set in the loop-gap resonator of the ESR spectrometer. Loop-gap type resonators are unsuitable for measuring radicals in the skin of experimental animals, because they detect radicals distributed throughout the body. By contrast, a surface-coil-type resonator (surface resonator) has been used

to measure radicals in limited positions, such as the skin (Bacic et al. 1989; Swartz et al. 1994; Kuppusamy et al. 1998; He et al. 2001; Fuchs et al. 1997). Furthermore, ESR measurement with a surface resonator does not interfere with light irradiation, since only the single-turn coil portion of the resonator is attached to the skin.

This study used ESR with a surface resonator to detect *in vivo* ROS generation with light exposure in the skin of griseofulvin-induced protoporphyria model mice, a standard model for EPP (Gschnait et al. 1975; Konrad et al. 1975; Wolff et al. 1975; Plosch et al. 2002).

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P.11 Acne Models

P.11.1 Activity on Sebaceous Glands of Rats

PURPOSE AND RATIONALE

Bioassays for topical antiandrogens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965), and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. Early transformations, which take place in the morphology of the organelles in sebaceous cells, can be observed by electron microscopy. In the cyto-

plasm of intermediate cells a large number of vesicular elements derived from the smooth endoplasmic reticulum is formed, participating in the synthesis of lipids which appear as droplets of varying size (Karasek 1968; Morohashi 1968). Following an increase of lipid droplets, the cells increase in size, become totally differentiated and are pushed towards the apex of the gland where they break up and release their content (sebum) into the infundibulum. This effect is used for morphometric evaluation of topical anti-androgens.

PROCEDURE

Groups of 5 adult male Sprague-Dawley rats weighing 180–220 g are shaved in the interscapular area. Twenty-four hours later, the test preparation or the standard (cyproterone acetate) is applied locally to the shaved area at increasing doses (0.05, 0.5, and 5 mg/cm²) in 20 µl ethanol. The treatment is continued for three weeks. Controls receive ethanol only. The animals are sacrificed 24 h after the last administration. Pieces of skin from the interscapular region are excised and processed for evaluation by electron microscopy. The volume density of the smooth endoplasmic reticulum vesicles is measured.

EVALUATION

Dose-response curves are established for volume density of vesicles of the smooth endoplasmic reticulum after various doses of antiandrogen and testosterone standard in order to calculate activity ratios. Statistical comparison with untreated controls allows calculation of threshold doses.

MODIFICATIONS OF THE METHOD

Ebling and Petrow (1993) tested 19-Aldehyde-4-androstene-3,17-dione, an estrogen precursor that inhibits sebaceous secretion in ovariectomized testosterone-treated rats.

De Young et al. (1984, 1985) described intradermal injection of *Propionibacterium acnes* as a model of inflammation relevant to acne. Intradermal injection of killed *P. acnes* into the rat ear induces a chronic acne-like inflammation characterized by edema and cell infiltration of several months' duration, formation of comedones, hypersensitization, and transepithelial elimination.

Shamoto et al. (1999) investigated the dermal histology and the regional draining superficial lymph node of a new mutant strain of hairless rats (ISh). The homozygote ISh rat was characterized as having naked and wrinkled skin. The comedo-like casts in the skin resembled human acne.

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P.11.2

Activity on Sebaceous Glands of the Fuzzy Rat

PURPOSE AND RATIONALE

Fuzzy rats, a genetic mutant between hairless and haired albino rats, exhibit sexual dimorphism in the skin (Ferguson et al. 1979). A brown-colored, thick seborrhoeic coating on the entire back is characteristic of male rats while the female rats show clear skin with white fuzzy hairs. In male rats the hair follicles resemble human sebaceous follicles associated with hyperplastic glandular lobules, dilatation and micro-comedo (Plewig and Kligman 1975). Castration caused a reduction in size of the sebaceous glands and ducts, and a testosterone implant in castrated rats resurrected glandular hyperplasia and ductal dilatation (Uno et al. 1990).

This rodent model for androgen-dependent hyperplasia of the sebaceous glands is useful for the study of many pharmacological aspects, comprising the rate of percutaneous absorption, stability, and affinity to target organs of the testing compounds and selection of adequate vehicles for topical application (Ye et al. 1997).

PROCEDURE

Peripubertal male fuzzy rats at an age of 25 days weighing about 40 g are kept in single cages at a temperature of 24°C and a 12-h light-and-dark cycle. They are divided into several groups for local treatment with 5 α -reductase inhibitors or androgen receptor antagonists. One group serving as control is castrated at an age of 25 days in anesthesia. Approximately 0.5 ml of a 1% solution of test compounds dissolved in 30% propylene glycol, 50% alcohol and 20% water are applied to the other groups in a 4 × 4 cm area on the lower back once per day 5 days per week. Vehicle solution alone is applied to the control and castrate groups. After 8 weeks of treatment, the animals are euthanized by anesthesia with ketamine HCl, 100 g/ml, xylazine HCl, 120 g/ml, acepromazine, 5 g/ml, 1 ml/kg intramuscularly.

Photographs of the backs are taken pre-treatment and every 2 weeks after the start of treatment.

Eight weeks after starting treatment, the animals in all groups are given an intraperitoneal injection of 200 mg/kg bromodeoxyuridine 2 h before euthanasia. A blood sample is then collected from the right ventricle of the heart under anesthesia with the ketamine-xylazine-acepromazine mixture. After euthanasia with an overdose of the same anesthesia solution, fresh skin tissues are taken by a punch (4 mm diameter) from the marked area of the back and immediately incubated with EDTA solution for the split-skin preparation. The rest of the skin in the marked area is cut and fixed with 10% neutral buffered formalin solution for morphometric analysis of the sebaceous glandular lobules. The ventral lobes of the prostate are dissected and the weight is measured.

For morphometric analysis of the sebaceous glands, the size of sebaceous glandular lobules is first determined. Using formalin-fixed skin, small skin samples are taken by punch (4 mm diameter) and serial frozen sections (40 μ m thick), horizontal to the epidermal surface, are cut and collected in water-filled wells. Four to five free-floating sections containing sebaceous glandular lobules are selected and stained with 1% osmium and 2.5% potassium dichromate solution for 2–3 min. The sections are washed with distilled water and mounted on a glass slide with aqua mount.

Darkly stained globular lobules are distinctively seen under the microscope or on microvideo images. The largest lobular area in each glandular image is measured by a computer-assisted microimage apparatus, using a program of image analysis.

Split preparations of the pilosebaceous organ are used to measure the size of the sebaceous lobule and duct, and the number of DNA synthesis sebocytes. Fresh skin samples, 4 mm punched, are incubated with 17 mM EDTA in phosphate buffer (0.1 M, pH 7.4) for 2 h at 27°C. The pilosebaceous organs attached to the epidermis are manually split from the dermis. Following fixation with 10% buffered formalin, free-floating split tissues are stained by the immunocytochemical method, using a monoclonal antibody against bromodeoxyuridine with the avidin-biotin complex method.

On viewing the split tissue under a stereomicroscope, the sebaceous glandular lobules with a duct attached to the follicular shaft are clearly visible. After mounting on a slide glass, these *in situ* images of the sebaceous glands are observed on a computer monitor with a microvideo apparatus, and the area of the lobes, the diameter of the duct and number of bromodeoxyuridine-stained nuclei in the sebocytes are measured.

EVALUATION

Group data are expressed as means \pm standard error. The results are analyzed using the *t*-test.

MODIFICATIONS OF THE METHOD

Marit et al. (1995) presented anatomical and physiological parameters of the fuzzy rat, collected for each sex at five ages, including histological and clinical biochemical profiles, organ and body weights, and a characterization of gross and histopathological findings.

Yourick and Bronaugh (2000) studied percutaneous penetration and metabolism of 2-nitro-*p*-phenylendiamine in human and fuzzy rat skin.

Salcido et al. (1995) described an animal model and computer-controlled surface pressure delivery system for the production of pressure ulcers. A method for inducing dermal pressure lesions on the fuzzy rat was developed using a computer-controlled displacement column, which produced a constant tissue interface pressure.

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P.11.3

Activity on Ear Sebaceous Glands of Syrian Hamsters

PURPOSE AND RATIONALE

The ear sebaceous glands of the Syrian hamster have been proposed as a model system for human sebaceous glands because of the similarities in morphology and in turnover time (Hamilton and Montagna 1950; Plewig and Luderschmidt 1977). As an androgen-sensitive structure, the ear sebaceous glands have been used to determine the effect of antiandrogenic compounds by the use of histoplanimetry on projections of sagittal sections of the ear (Luderschmidt and Plewig 1977). Matias and Orentreich (1983) developed a stripped skin planimetric method to measure the ear sebaceous gland areas in Syrian golden hamsters.

Seki et al. (1995) determined the effects of topically applied spironolactone on androgen stimulated sebaceous glands in the hamster pinna.

PROCEDURE

Adult female Syrian golden hamsters, 9–10 weeks of age and 110–120 g in weight, are kept at constant temperature and humidity and fed commercially prepared hamster food and water. The hamsters are divided at random into three groups. Testosterone propionate (80 µg dissolved in 1 ml sesame oil) is administered to two groups every other day over a two weeks period to stimulate androgen responses. Hamsters in the third group are injected with 1 ml sesame oil only as controls. The hamsters receive once daily applications of 0.1 g of a colorless clear hydrophilic gel preparation of 5% spironolactone, containing ethanol and isopropyl alcohol on the ventral side of the right pinna for two

weeks. The left side is left untreated as control. On day 15, 4 mm punch biopsy specimens from each hamster are obtained from the central region of the bilateral pinnae where sebaceous glands are most developed.

The sebaceous gland size is measured by the whole mount technique (Motoyoshi 1988). After the biopsy specimens are immersed in physiological saline at 4°C for 18 h, cartilages are removed. Then, each specimen is immersed in 2 N NaBr at 37°C for one hour, and the epidermis is peeled off with fine forceps. The obtained dermis sheet containing sebaceous glands is stained with Susan III and mounted on a glass slide.

EVALUATION

An area of five or more sebaceous glands, including all the sebaceous acini attached to one pilosebaceous unit in each biopsy specimen, is measured with a computerized image analyzer (Olympus CIA-102) and shown as a mean value \pm standard deviation ($\text{mm}^2/100$). The values are compared using Student's paired *t*-test.

MODIFICATIONS OF THE METHOD

Gollnick (1990, 1992) evaluated azelaic acid proposed for acne treatment, using comedo formation in the hamster ear model and recommended it as a new substance in the spectrum of antiacne agents.

Matias and Gaillard (1995) studied the local inhibition of sebaceous gland growth in the ventral ear pinna of sexually mature male Syrian hamsters by topically applied androgen receptor inhibitors.

Seki and Morohashi (1993) investigated the effect of some alkaloids, flavonoids and triterpenoids on the lipogenesis of sebaceous glands of the hamster ear. Lipogenesis was assayed by determining ^{14}C incorporation into sebaceous lipids extracted from the sebaceous glands, which were incubated with ^{14}C -acetate.

Morgan et al. (1993) studied the occurrence of zinc-induced synthesis of metallothionein in skin after topical application of the anti-acne drug Zineryt lotion in hamster ears. The dinitrophenyl hapten-sandwich immunohistochemical method involving a monoclonal anti-metallothionein antibody was used to detect and localize zinc-binding metallothionein in the treated and untreated hamster skin.

Burkhart et al. (2000) studied the effects of benzoyl peroxide on lipogenesis in sebaceous glands using the flank organs of female golden Syrian hamsters.

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- glands are reconstructed from their serial histological sections using a computer-image analysis system.

EVALUATION

The volumes of the sebaceous glands, the number of acini, and the volume of individual acini are compared between groups.

MODIFICATIONS OF THE METHOD

Motoyoshi (1983b) studied the correlation between surface microscopy and dermal histology in tetradecane-induced comedones in rabbit ear skin.

Kligman and Kligman (1994) used the rabbit ear to assay the comedogenic potentialities of an array of known tumorigens. Complete carcinogens and some tumor promoters were invariably strongly comedogenic at concentrations of 1.0% and below. The rabbit ear model was recommended to be an easy and reliable way to screen for carcinogenicity.

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P.11.4

Activity on Ear Sebaceous Glands of Rabbits

PURPOSE AND RATIONALE

Several authors used the rabbit ear model to study comedo formation (Weirich and Longauer 1974; Mills and Kligman 1975; Motoyoshi 1983a; Kligman 1989; Ito et al. 1985, 1991; Maeda 1991) in order to assess the comedogenicity of cosmetics, toiletries and drugs and to evaluate potential anti-acne drugs. Sebaceous follicles in the inner surface of rabbit ears are sensitive to many substances called comedogenes, which, when applied topically, induce comedo formation. This comedo induction takes place after about 2 weeks of repeated topical application of a chemical comedogen such as 1% coal tar, 50% oleic acid, or 50% tetradecane.

PROCEDURE

Male rabbits weighing 2.5–3.5 kg are used. Tetradecane, testosterone, and dimethyl sulfoxide are separately injected on rabbit pinna once a day for 4 weeks. The pinnae are biopsied on days 1, 3, 7, and 28. Untreated pinnae and squalene-treated pinnae serve as controls. Three-dimensional images of sebaceous

P.11.5

Activity on the Hamster Flank Organ

PURPOSE AND RATIONALE

The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species,

these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemic or topical anti-androgens.

PROCEDURE

Female Syrian golden hamsters (*Mesocricetus auratus*), weighing 80–110 g are kept at constant temperature on a commercial diet and water ad libitum. They are castrated 24 h prior to the experiment. The costovertebral region is shaved; the horny layer is stripped the day prior to the test and then every 3 days. For stimulation, animals receive a subcutaneous dose of 250 µg testosterone propionate in 25 µl peanut oil in the dorsal neck fold, for three weeks (weekdays only or continually). On the same days, the anti-androgen dissolved in water/ethanol (1:4, v/v) is applied locally to the left flank organ at increasing doses, using a micropipette under a continuous air stream to enhance the evaporation of the solvent. At least 5 animals are used per group. Controls receive vehicle only, and another group, testosterone propionate only. After treatment the animals are sacrificed under ether anesthesia. The 2 major perpendicular axes of the pigmented spot overlying each flank organ are measured and multiplied to obtain the surface area index. The flank organs are excised and divided into two halves along the major axis, immediately fixed in 10% formalin and embedded in paraffin. The 5 µm-thick sections are stained with haematoxylin-eosin. Sebaceous glands and hairs are measured in the first 2 sections of each half of the specimen, using a semiautomatic computerized image analyzer. The sebaceous gland area in each field is quantified in square millimeters. The diameter of all the hair under each flank organ is measured in micrometers.

EVALUATION

The local (topical) anti-androgenic activity of the test compound is estimated by the ability to inhibit the effects in the ipsilateral treated flank organ whereas the systemic activity is evaluated by the inhibition on the untreated contralateral flank organ. The values for surface area index, sebaceous gland area, and average of diameter of the hairs on the flank organ of the treated left and the untreated right side are compared for anti-androgen-treated animals with controls using two-way analysis of variance and *t*-test.

MODIFICATIONS OF THE METHOD

Wuest and Lucky (1989) studied the differential effect of testosterone on pigmented spot, sebaceous glands and hair follicles in the Syrian hamster flank organ.

Noto et al. (1991) quantified the antiandrogenic activity of topically applied canrenoic acid in the hamster flank organ. The flank organs of female Syrian hamsters were stimulated by subcutaneous administration of testosterone propionate. Sebaceous glands and hair follicles were measured by a computerized image analyzer. Using this method, the same authors (Noto et al. 1992) compared the activity of some topical antiandrogens.

Aricò et al. (1993) found no antiandrogenic effects of topical bifonazole on sebaceous glands and hair in the hamster flank organ.

Lucky et al. (1995) studied the autoradiographic localization of tritiated dihydrotestosterone in the flank organ of the albino hamster.

Foreman et al. (1984) found that in the hairless hamster, progesterone can antagonize dihydrotestosterone-mediated hypertrophy of the sebaceous gland.

CRITICAL ASSESSMENT OF THE METHOD

The relevance of the hamster flank organ model to man has been challenged by Franz et al. (1989).

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P.11.6

Activity on the Skin of the Rhino Mouse

PURPOSE AND RATIONALE

The Rhino mouse has been widely used as an experimental acne model to evaluate topically active comedolytic and anti-keratinizing agents (Kligman and Kligman 1979; Ashton et al. 1984; Mezick et al. 1984; Chatelus et al. 1989; Bernerd et al. 1991; Bouclier et al. 1991; Tramposch et al. 1992; Zheng et al. 1993; Sundberg 1994; Fort-Lacoste et al. 1999).

Rhino mice are hairless mutants with a rhinoceros-like appearance, which carry the rhino gene, a recessive allele of the hairless gene ($hr^{rh}hr^{rh}$) (Howard 1941). This recessive mutation on chromosome 14 results in a mouse with wrinkled skin devoid of body hair by age of 25 days. At that time, the end of the first hair cycle, the follicular papillae fail to follow the regressing hair follicles and become isolated in the dermis. The papillae do not re-associate with the follicular epithelium to initiate a new hair follicle cycle. The upper remnants of the hair follicles are filled with sloughed, cornified cells and form utriculi with a small sebaceous gland at their base, resembling an open comedone. The Rhino skin becomes progressively loose, forming folds and ridges, due to the expansion of the surface, secondary to abortive hair follicles filling with cornified debris. The utriculi progressively enlarge, forming pillory cysts (pseudocomedones), which are dilated follicular infundibula filled with cornified debris (Mann 1971). The dermal cysts of the rhino mouse develop into unopened sebaceous glands (Bernerd et al. 1996).

Seiberg et al. (1997) studied the effects of trypsin on apoptosis, utriculi size and skin elasticity in the rhino mouse. González et al. (2000) measured the effect of graduated local doses of all-*trans* retinoic acid applied

to the skin of rhino mice with fluorescence excitation spectroscopy and compared the data with histological findings.

PROCEDURE

Seven-weeks old female rhino mice ($hr^{rh}hr^{rh}$) obtained from Charles River Laboratories are divided into groups receiving graduated doses of test compound (all-*trans* retinoic acid) dissolved in an ethanol/propylene glycol mixture (70:30) or the solvent as controls. The solution is applied once per day in doses of 100 μ l on the entire dorsal skin, for 5 consecutive days each week, for a total period of 2 weeks. Animals in all groups are sacrificed 24 h after the last treatment and biopsies are obtained immediately following sacrifice. Fluorescence excitation spectra are collected from dorsal skin on a daily basis during treatment. During the measurements, the animals are sedated with inhalation of methoxyflurane.

Fluorescence excitation spectra are obtained *in vivo* with a fluorescence spectrophotometer (SkinSkan, SPEX, Edison NJ) equipped with a 450 W Xenon lamp, double monochromators on the excitation and emission, a photomultiplier detector (R928P, Hamamatsu, Japan) connected to a single photon counting system and a bifurcated quartz fiber bundle (Model 1950; SPEX Ind., Edison, NJ) for light delivery and collection. The individual fibers are 100 μ m in diameter and are randomly arranged to form a bundle 6 mm in diameter. The resolution is 4 nm, the intensity of the excitation radiation is in the range of 1–20 mW/cm², and the total delivered radiation dose is below the erythema threshold. Each fluorescence measurement consists of a set of eight serial excitation spectra collected by positioning the emission monochromator from 340 to 480 nm in increments of 20 nm and scanning the excitation monochromator from 260 to within 20 nm of the emission monochromator setting. Light from the excitation monochromator is focused into one leg of the bifurcated fiber bundle. The other leg of the fiber bundle is focused into the input of the emission monochromator. The joined end of the fiber bundle is brought into direct contact with the skin site measured. Care is taken to clean properly the fiber bundle end with an alcohol swab between measurements on different animals and that gentle pressure is applied to the animals during measurement.

Significant changes are observed in the fluorescence spectra as a result of the application of comedolytic agents. The first peak, located approximately at

295 nm, which is related to tryptophan, increases significantly, whereas the second peak at 340–370 nm, which is attributed to collagen links, exhibits a dramatic decrease.

For histology, skin samples approximately 5 mm in diameter are obtained from the skin of each animal following sacrifice, from the mid-line of the anterior portion of the skin. All specimens are fixed in 10% buffered formaldehyde for 24 h, and later embedded in paraffin. Tissue sections 5 μ m thick are cut perpendicularly to the epidermal surface and stained with hematoxylin and eosin for light microscopic examination. For each animal, the epidermal and dermal thickness, utriculi diameter, and number of capillaries containing more than 5 erythrocytes are assessed with a micrometer eyepiece adapted to a microscope (Leitz SM-LUX, Ernst Leitz, Wetzlar, Germany) using a 40 \times objective for the measurement of epidermal and dermal thickness, and utriculi diameter, and a 20 \times objective for the count of dilated capillaries.

EVALUATION

Time-response curves and dose-response curves of the changes of fluorescence spectra are established compared with the changes of histological data.

MODIFICATIONS OF THE METHOD

In addition to image analysis, Seiberg et al. (1997) tested the effects of trypsin on skin elasticity and elastin expression in the Rhino mouse.

Beehler et al. (1995) studied gene expression of retinoic acid receptors and cellular retinoic acid-binding proteins in rhino and hairless mouse skin.

Imakado et al. (1995) found that targeting expression of a dominant-negative retinoic acid receptor (RAR) mutant in the epidermis of transgenic mice results in loss of barrier function.

Feng et al. (1997) reported that suprabasal expression of a dominant-negative RXR alpha mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids.

González et al. (1997) investigated DNA ploidy changes in rhino mouse skin after treatment with *all-trans* retinoic acid and retinol.

Petersen et al. (1984) developed an animal model using human face skin onto the nude mouse to study human sebaceous glands. The effects of androgens were evaluated.

Lesnik et al. (1992) reviewed agents that cause enlargement of sebaceous glands in hairless mice.

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P.11.7

Activity on the Skin of the Mexican Hairless Dog

PURPOSE AND RATIONALE

The Mexican hairless dog has been recommended as a model for the comedolytic and morphogenic activity of retinoids and other anti-acne agents (Loux et al. 1974; Schwartzman et al. 1996; Kimura and Doi 1996). A semilethal mutation of an autosomal dominant 'L' gene produces an animal with multiple developmental defects, including poor dentition, early degeneration of the thymus, hairlessness and numerous comedones (Yankell et al. 1970a; González-Diddi et al. 1971; Goto et al. 1987; Fukuda et al. 1991). The Mexican hairless dog is nearly bald, with sparse, flimsy hair most abundant on the limbs and head. In most hairy species, the primary hair is surrounded by 7 to 10 accessory hairs. In this species, the follicles are mainly single and not in clusters. The skin is tan-colored. A brownish, waxy material unevenly coats the surface; this can be removed easily by soap and lipid solvents. A great number of comedones cover the surface, and these are nearly all of the open variety. The majority is rather small (a few millimeters in diameter), and may be viewed as horn-filled shallow invaginations. Scattered among these, especially on the face, neck and thighs, are larger, black-tipped open comedones. These are hard, deep, horny impactions, which are expelled with difficulty. A few small, closed comedones occur on the neck and lateral aspect of the chest.

Papulopustules are rare, and are found mainly on the metacarpus and metatarsus. They apparently do not develop from the rupture of comedones as in humans. Thus, this animal mimics human acne only with regard to comedones. These, unlike the human variety, do not originate from pre-existing sebaceous follicles. They arise *de novo*, and completely lack sebaceous glands.

PROCEDURE

About 1-year-old female animals are used. Areas of 4–5 cm² on the dorsolateral aspects of the trunk

are treated once daily (5 working days) for 14 weeks with test formulations or tretinoin formulations (0.025–0.1%) as standard.

Biopsies are taken from each site under local anesthesia at 5 weeks and again at 14 weeks.

EVALUATION

The clinical picture and the histology of biopsies are compared before, during, and after treatment.

MODIFICATIONS OF THE METHOD

Yankell et al. (1970b) used the Mexican hairless dog for sunscreen recovery studies.

Hunziker et al. (1978) compared percutaneous penetration of benzoic acid, progesterone and testosterone between Mexican hairless dogs and man.

Matsumura et al. (1992) described a burn wound-healing model in the hairless descendant of the Mexican hairless dog.

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P.11.8

In Vitro Sebocyte Model

PURPOSE AND RATIONALE

Cultured human sebocytes have been used by several authors to study sebum formation and to evaluate po-

tential anti-acne drugs (Zouboulis et al. 1991a, b, 1993, 1994, 1998; Rosenfield 1989; Doran and Shapiro 1990; Doran et al. 1991; Akamatsu et al. 1993; Guy et al. 1996a, b; Rosenfield et al. 1998; Wauben-Penris et al. 1998; Tsukada et al. 2000; Fritsch et al. 2001).

PROCEDURE

Human sebaceous glands are isolated from facial skin and seeded on monolayer 3T3 cells (Xia et al. 1989). Primary sebocyte cultures are derived from the periphery of the gland lobules and are maintained to confluence before subcultivation. All experiments are performed using secondary sebocyte cultures, which consist of cells undergoing sebocytic differentiation.

Human sebocytes are seeded in 96-well culture plates at a concentration of 10^4 cells/well and are left to attach for 2 days at 37°C with 5% CO_2 in culture medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) supplemented with 8% fetal calf serum, 2% human serum, 10 ng/ml epidermal growth factor, 10^{-9} M cholera toxin, 3.4 mM L-glutamine, 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The medium is then aspirated, and serum-free keratinocyte basal medium (KBM) (Clonetics, San Diego, CA) without additives, supplemented with steroids, e.g., testosterone (10^{-8} – 10^{-5} M), 5 α -dihydrotestosterone (10^{-8} – 10^{-5} M), or spironolactone (10^{-12} – 10^{-7} M) or their combinations are added to 6 wells at each concentration. KBM is concomitantly added to another 6 wells serving as controls. The plates are incubated at 37°C for 10 days before evaluation. KBM with and without compounds is changed every 2 days.

Cell numbers of treated human sebocytes in 96-well culture plates are assessed over 9 days by counting single-cell suspensions in Neubauer chambers and compared with the absolute fluorescence units (AUF) of parallel wells obtained using the 4-methylumbelliferyl heptanoate (MUH)-fluorescence assay (Stadler et al. 1989). This assay is based on the hydrolysis of the fluorogenic substrate MUH by esterases of proliferating cells. A stock solution of 10 mg/ml MUH is prepared in DMSO and kept frozen at -20°C until use. On the day of assessment, KBM is removed, and the cells are washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (pH 7.2). The MUH stock solution is diluted in phosphate-buffered saline up to 100 $\mu\text{g}/\text{ml}$, and 100 μl of the final solution is added to each well. The plates are then incubated for 30 min at 37°C and read automatically on a Titertek Fluoroscan II (Flow, Meckenheim, Germany).

EVALUATION

The results are given as absolute fluorescence units using 355-nm excitation and 460-nm emission filters. Statistical significance of the differences between the means is assessed by Student's *t*-test.

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P.12 Skin Mycosis

P.12.1

General Considerations

PURPOSE AND RATIONALE

Fungal infections of the skin account for a large number of consultations to general practitioners and dermatologists. They are caused by dermatophytes, such as *Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Epidermophyton floccosum*, *Microsporum gypseum*, and *Trichophyton verrucosum*. (Sinski and Kelley 1991).

Most fungal skin infections, such as tinea pedis and tinea cruris, respond to topical therapy, although widespread or chronic infections that do not respond to local measures may require systemic treatment, for example with griseofulvin, ketoconazole or terbinafine. Traditional topical products such as compound benzoic acid ointment (Whitfield's ointment) and tolnaftate preparations have largely been superseded by other topical-active antifungal agents, such as the imidazoles (e. g. clotrimazole, miconazole or econazole) or hydroxypyridones (e. g. ciclopirox), which are well tolerated and rapidly effective (Gupta et al. 1994). Fungal infections also affect the appendices, such as hair and nails. To evaluate antifungal agents not only the antimicrobial spectrum, but also the penetration plays a decisive role.

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P.12.2

In Vitro Inhibitory Activity

PURPOSE AND RATIONALE

In vitro tests are performed to investigate whether the test compound in comparison to standards covers the most relevant pathogens of dermal mycoses. The test

conditions are rendered more difficult by addition of protein since the main infection site for fungi is the horny layer of the epidermis, which has a high protein content.

PROCEDURE

The studies are performed by means of conventional serial dilution procedures in test medium without and with addition of 4% bovine albumin. The test medium is Sabouraud dextrose broth containing 1% Neopeptone Difco (Difco Laboratories, Detroit, Mich., USA) and 2% glucose. The basic medium is sterilized in an autoclave at 121°C for 15 min. The pH is adjusted to 6.5 with 1 N NaOH. The medium containing albumin is sterilized by filtration through a membrane filter. For preparation of the test series, the inhibiting substances are dissolved in methanol and then rapidly diluted with slightly warmed test medium, so that series with a continuous dilution factor of 2 are obtained: 125–0.03 µg/ml in medium without protein, 500–1 µg/ml in medium with protein. Each test tube contains 3 ml. The test organisms are various strains of dermatophytes (*Trichophy-tum mentagrophytes*, *T. rubrum*, *T. verrucosum*, *T. equinum*, *T. gallinum*, *Microsporum canis*, *Microsporum gypseum*) and yeasts (*Candida albicans*, *Ca. tropicalis*, *Ca. pseudotropicalis*, *Ca. krusei*, *Ca. parapsilosis*, *Ca. lipolytica*, *Ca. brumpti*, *Ca. utilis*, *Torulopsis glabrata*). The organisms are pre-cultured on a modified Grütz agar at 28°C for periods of 1–4 weeks. The suspensions are adjusted by photometry that about 10⁵ microconidia of dermatophytes and 10⁴ yeast cells per ml are obtained in each inoculated test tube. The minimal inhibitory concentrations are measured after 14 days incubation at 28°C.

EVALUATION

The percentage of strains of *Trichophy-tum mentagrophytes*, *Trichophy-tum rubrum*, and *Candida albicans* is plotted against dilution steps for each test compound with and without albumin and IC₅₀ values are calculated.

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P.12.3**In Vivo Activity in the Guinea Pig Trichophytosis Model****PURPOSE AND RATIONALE**

The guinea pig trichophytosis model has been used by several authors to evaluate antimycotic compounds (Millberger and Gillert 1973; Dittmar et al. 1981; Plempel et al. 1983; Petranyi et al. 1987; Schaude et al. 1990; Garcia Rafanell et al. 1992; Arika et al. 1993).

PROCEDURE

Male albino guinea pigs (Pirbright White), bred mycosis-free, weighing 450–550 g are fed Altromin pellets and tap water ad libitum. On both sides of the back, areas of 5 × 12 cm are shorn to a fur length of 1 mm. Three areas with a diameter of 3 mm are inoculated with a pipette on either side. Per injection site, 10⁴ spores of *Trichophyton mentagrophytes* 2114 in 0.05 ml suspension in physiological saline solution are inoculated. Three days after inoculation, infections with redding and scale formations are observed. From days 3–7 after the infection, 1 ml of the test preparation or standard is applied onto the right animal sides and rubbed in once daily. The diameters (mm) of all alopecias are measured with a ruler 3.5 weeks after the infection.

EVALUATION

The values of alopecias, separated according to the treated group and animal side, are determined and statistically evaluated using Duncan's new multiple range test.

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P.12.4**Skin Penetration****PURPOSE AND RATIONALE**

Cutaneous pathogenic fungi like dermatophytes or *Candida* ssp. present a particular affinity for the stratum corneum. Therefore the bioavailability of antimycotic drugs in the thick horny layer of the skin is an important element of treatment success. These studies can be performed in human cadaver skin (Dittmar 1981; Kligmann et al. 1987; Hänel et al. 1988)

PROCEDURE

Skin pieces, measuring 10 × 12 cm are removed from the back of corpses not later than 24 h after death. The subcutis is removed and the skin piece divided 3 to 5 parts. The edges (4 mm) of each individual piece are then taped with Tesa-film (adhesive tape) and the remaining surface is treated with the antimycotic compound. 0.05 ml of compound preparation or solution is rubbed into the skin for 10 s. For a period of one h, the pieces are placed on water agar, allowing for free admission of air. After this time, the compound is carefully wiped off with filter paper and water.

The skin area treated with the antimycotic is then stripped with Tesa-film tape as follows: (A) one third of the area remains unstripped; (B) one third of the area is stripped three times; (C) one third of the area is stripped six times. Then each piece of skin is placed into a Petri dish, which is heated in a water bath to 52°C for 15 min. The epidermis is removed from all the skin pieces, which are then placed on slides (with the base turned upwards) and put into the upper parts of plastic Petri dishes. Each of the epidermis pieces is inoculated in 20 sites with a microconidia suspension of the dermatophyte *Trichophyton mentagrophytes* 109-FHM 1a. The spores had been rinsed off from three-week-old slant cultures, using distilled water. Approximately 50–100 viable spores are deposited at each inoculation site.

The upper parts of the Petri dishes (containing the slides with the epidermis pieces) are then covered with the bottom parts of Petri dishes, which contain 2% aqueous agar for producing a wet chamber. Holes in the covers serve to avoid excess humidity in the chamber. The chambers are incubated at 28°C for 5 days. The fungal growth is recorded daily according to a three points score system.

EVALUATION

Growth is evaluated by visual scoring from days 2–5 after inoculation and calculation of AUCs from growth curves.

Inhibition values (%) are calculated according to the formula:

$$\text{inhibition} = \frac{\text{growth on controls} - \text{growth after treatment}}{\text{growth on controls}} \times 100$$

MODIFICATIONS OF THE METHOD

Ceschin-Roques et al. (1991) performed penetration studies on excised skin from the back of slaughtered pigs.

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P.13**Biomechanics of Skin****P.13.1****General Considerations**

Several attempts were made to describe the mechanical properties of skin by mathematical models (Ridge and Wright 1965; Harkness 1971; Hirsch and Sonnerup 1968; Jamison et al. 1968; Viidik 1968, 1969, 1973, 1979; Frisén et al. 1969a, b; Veronda and Westman 1970; Danielson 1973; Soong and Huang 1973; Wilkes et al. 1973; Jenkins and Little 1974; Lanir and Fung 1974; Vogel 1976, 1986; Barbanel and Evans 1981; Barbanel et al. 1978; Lanir 1979; Barbanel and Payne 1980; Burlin 1980, 1981; Fung 1981; Sanjeevi 1982; Potts and Breuer 1983).

Most of these authors used models derived from studies in polymers (Ferry 1970). The simplest mechanical model analogous to a viscoelastic system is a spring combined with a dashpot, either in series

(**Maxwell element**) or in parallel (**Voigt or Kelvin element**). Combinations of these elements were used to explain the mechanical phenomena in connective tissue, such as stress-strain behavior, relaxation and mechanical recovery, hysteresis and creep phenomena (Jamison et al. 1968; Frisén et al. 1969a, b; Hirsch and Sonnerup 1968; Vogel 1976, 1993; Riedl and Nemetscheck 1977; Vogel and Hilgner 1979; Viidik 1968, 1969, 1973, 1977, 1979). Larrabee (1986), Larrabee and Sutton (1986).

Larrabee and Galt (1986) reviewed the theoretical and experimental mechanics of skin and soft tissue and proposed a mathematical model of skin deformation based on the finite element method. A finite element based method to determine the properties of planar soft tissue was also described by Flynn et al. (1998).

Unfortunately none of these models has been found to be sufficient to describe all properties of human and animal skin including the mechanical history before measurement and the time-dependence during measurement. There is no comprehensive and unequivocally accepted model to describe completely the biorheology of skin. Therefore, several methods are used in order to get insight into the physical properties of skin.

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P.13.2

In Vitro (ex Vivo) Experiments

P.13.2.1

Stress-Strain Behavior

P.13.2.1.1

Measurement of Skin Thickness, Ultimate Load, Tensile Strength, Ultimate Strain and Modulus of Elasticity

PURPOSE AND RATIONALE

Animal experiments are preferable when studying the biomechanical properties of the dermis, since only in animals can the values at higher extension degrees be studied, *ex vivo* or *in vivo* under anesthesia, whereas studies in humans are limited by pain threshold or to tests in cadaver skin. Skin thickness, ultimate load, tensile strength, ultimate strain and ultimate modulus of elasticity are the most informative parameters, which describe the mechanical properties of the dermis.

PROCEDURE

Groups of at least 10 male Sprague-Dawley rats with an initial weight of 120 ± 5 g are treated subcutaneously or orally with test drugs or saline. The duration of treatment is usually 5 days; however, for special studies treatment can be prolonged up to 3 months. The animals are sacrificed under anesthesia. The back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness. In this way, **skin thickness** can be measured reliably by calipers with an accuracy of 0.1 mm. Perpendicular to the body axis two dumbbell shaped specimens with a width of 4 mm in the middle of the sample are punched out (Vogel 1969, 1970, 1989, 1993a). The samples are kept in Petri dishes until testing at room temperature on filter paper soaked with saline solution. The specimens are fixed between the clamps of an INSTRON instrument at a gauge length of 30 mm. All measurements are carried out within at least 1 h. For long lasting test procedures, such as relaxation or cyclic loading, the samples are wrapped with saline soaked filter paper (Vogel 1976a, b 1989, 1993a, b).

Stress-strain curves are registered at an extension rate of 5 cm/min, showing a characteristic shape. During low strain values, there is a gradual increase of load; the curve has a concave part. The stress-strain curve ascends according to an exponential func-

tion (Vogel and Hilgner 1977). Afterwards an almost straight part is reached indicating the dependence on Hook's law. At this part the **ultimate modulus of elasticity** (Young's modulus) can be calculated (increase of load divided by the cross sectional area). Then some yielding of the curve occurs which ends in a sudden break of the specimen. This point indicates **ultimate strain** and **ultimate load**.

EVALUATION

From ultimate load divided by the cross sectional area (specimen width times original skin thickness measured at the beginning of the experiment), **tensile strength** can be calculated. The mean values of skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity of treated animals are compared with controls using ANOVA and Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The parameters: **skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity** are influenced by many factors, such as hormones and desmotropic drugs. The most pronounced changes are seen after glucocorticosteroids, both after systemic and local application (Vogel 1969, 1970, 1974a). Several studies were performed on age dependence of mechanical parameters in rat skin. Ultimate load, tensile strength and modulus of elasticity show a very sharp increase during puberty, a maximum at 12 months and a slight decrease thereafter (Vogel et al. 1970; Vogel 1976b, 1978, 1983, 1988, 1989, 1993a). Studies on age dependence show a similar pattern for skin thickness, ultimate load, tensile strength, ultimate extension, modulus of elasticity, hysteresis, relaxation, creep behavior and biochemical data both in animals and men (Holzmann et al. 1971; Vogel 1987a, b).

Therefore, extrapolations from animal studies to behavior of human skin are justified.

However, due to the anatomical conditions (haired skin in animals) the biomechanics of the epidermis can be studied better in human experiments than in animals.

MODIFICATIONS OF THE METHOD

Measurement of skin thickness, ultimate strain, ultimate load, tensile strength, and modulus of elasticity can be used in the evaluation of topical corticosteroids (Schröder et al. 1974; Alpermann et al. 1982; Vogel and Petri 1985).

Similarly, Töpert et al. (1990) measured skin atrophy and tensile strength of skin after 30 days local administration of corticosteroids in rats.

Oxlund and Manthorpe (1982) found an increase of strength and a decrease of extensibility of skin strips after long-term glucocorticoid treatment of rats. In agreement with studies by Vogel (1974b, 1978), these results were explained by a change in collagen cross-linking pattern.

Jørgensen et al. (1989) found a dose-dependent increase of mechanical strength in intact rat skin after treatment with biosynthetic human growth hormone.

Andreassen et al. (1981) studied the biomechanical properties of skin in rats with streptozotocin-induced diabetes. An increased stiffness and strength was found: maximal stiffness was increased by 20% and the strain rate at maximum stress was decreased by 10%.

Oxlund et al. (1980) found that the stiffness of rat skin was increased in the early postpartum period. This increase was also found in adrenalectomized animals.

Foutz et al. (1992) studied the effects of freezing on mechanical properties of rat skin.

Among desmotropic drugs, *lathyrogenic* compounds, such as amino-acetonitrile, and *D-penicillamine* decrease ultimate load, tensile strength without major influence on skin thickness (Vogel 1971a, b, 1974a). A decrease of the strength of skin strips in rats after treatment with D-penicillamine was also found by Fiedrich et al. (1975) and by Oxlund et al. (1984).

A dose-dependent increase of tensile strength in skin after treatment with was found with **non-steroidal anti-inflammatory drugs** (Vogel 1977).

Strain rate influences the values of ultimate load, tensile strength and modulus of elasticity, but not the effect of age and of corticosteroids (Vogel 1972b). Changes in tensile strength are correlated with the content of insoluble collagen (Vogel 1974b).

Fry et al. (1964) prepared skin rings from the lower part of the leg in rats and studied the age-dependence of the mechanical properties.

Nimni et al. (1966) measured tensile strength of excised skin samples in rabbits during aging.

Pan et al. (1998) studied ultrasound, viscoelastic and mechanical properties in rabbit skin, including stress relaxation, creep and Young's modulus as a function of strain.

Lofstrom et al. (1973) described circadian variations of tensile strength in the skin of two inbred strains of mice.

The effect of radiation therapy on mechanical properties of skin was studied in mice by Hutton et al. (1977) and by Spittle et al. (1980).

Schneider et al. (1988) measured tensiometric properties in guinea pig skin from flaps of normal dor-

sal skin and after implantation of an ovoid **tissue expander** filled for four days with saline.

Belkoff et al. (1995) studied the mechanical properties of skin in pigs after subcutaneous implantation and inflation of silicone tissue expanders.

Mustoe et al. (1989) compared the effects of a conventional tissue expansion regimen of 6 weeks with an accelerated regimen of 2 weeks in a model in dogs and measured skin thickness, elasticity, creep and stress relaxation.

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P.13.2.1.2

Measurement of Mechanical Properties at Low Extension Degrees

PURPOSE AND RATIONALE

At low strain values, stress-strain curves of skin samples show a gradual increase of load, the curve has a concave part. The stress-strain curve ascends according to an exponential function (Vogel and Hilgner 1977).

PROCEDURE

Groups of at least 10 male Sprague-Dawley rats with an initial weight of 120 ± 5 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia; the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out (Vogel 1969, 1970, 1981, 1989, 1993). The specimens are fixed between the clamps of an INSTRON instrument at a gauge length of 30 mm. An extension rate of 5 cm/min is chosen. Tension is registered at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80 and eventually 90% elongation. The registration is started with high sensitivity and switched to one tenth of the sensitivity in order to register the steep end of the curve.

EVALUATION

During registration with high sensitivity, the curve reaches an almost straight part. At this part, a tangent is drawn which is used for calculation of E_2 . The distance from the start to the cross-point of this tangent with the baseline is measured and denominated as extension until the first rise (D). The angle between the tangent and the baseline is halved. At the cross-point

with the stress-strain curve another tangent is drawn and used for calculation of E_1 . At the second part of the curve registered with low sensitivity a further tangent is drawn for calculation of E_3 , which is identical to the ultimate modulus of elasticity.

Mean values for E_1 , E_2 , E_3 , and ultimate stress of skin strips from treated animals are compared with controls using Student's t -test.

MODIFICATIONS OF THE METHOD

Belkoff and Haut (1991) developed a structural model to evaluate the changing microstructure of maturing rat skin.

Foutz et al. (1994) developed a phenomenological model to characterize the non-linear portion of stress-strain curves of rat skin.

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P.13.2.1.3

Step Phenomenon

PURPOSE AND RATIONALE

Analysis of the low parts of the stress-strain curve revealed a “step”-phenomenon (Vogel and Hilgner 1977, 1979a, b; Vogel 1988). If samples obtained perpendicular to the body axis are extended, a gradual increase of load is observed at low degrees of extension,

which is suddenly interrupted by a decrease of the registered curve. Then the curve increases again, being interrupted by a second or third step.

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out. The ends of the skin strips are fixed in the clamps of an INSTRON instrument resulting in a gauge length of 30 mm. The specimen is stretched with a strain rate of 50 mm/min. The first part of the stress-strain curve is registered with 10 times higher amplification than the second part. The steps are evaluated as follows.

EVALUATION

Tangents to the curves are drawn before and after each step. Then a vertical line is drawn in the middle of the step. The distance between the cross-points of this line with the tangents is measured and calculated as stress loss (ΔS). Furthermore, in the middle of each vertical line a horizontal line is drawn. The distance between the cross-point with the curves is measured and calculated as elongation due to the step (ΔE). Total stress loss is calculated by adding all values of ΔS and dividing by the number of specimens.

If this parameter is considered not only by itself but also in connection with tensile strength it can be calculated as percentage of ultimate stress (= % of breaking strength). In addition, the stress value, which would have been achieved without the step (= value S) and the elongation at which the step occurred (= value E) is registered. If stress loss at one step is multiplied with the elongation at this point, an indication of work loss is achieved (value ΔS times E). Taking into account the corresponding parameters indicating total work input (ultimate stress times ultimate strain), the relative work loss can be calculated.

Furthermore, elongation gain due to the steps is calculated by adding all values of ΔE and by dividing by the number of specimens.

Mean values of these parameters from treated animals are compared with controls using Student's t -test.

CRITICAL ASSESSMENT OF THE METHOD

The step phenomenon can be explained by the different orientation of collagen fibers in the dermis and by the presence of a muscular layer in rat skin. The muscular fibers are in a direction longitudinal to the body axis. If samples are obtained perpendicular to the body axis, the muscle bundles are cut transversally. In further studies the muscle layer was removed in one specimen and compared with a control still having the muscle layer. Investigation of the directional variation showed that the step phenomenon is mainly due to the muscular layer oriented longitudinal to the body axis and the connective tissue between the muscle bundles, whereas the anisotropic behavior of extensibility and ultimate strain is caused by the directional variation of the collagenous bundles in the dermis.

MODIFICATIONS OF THE METHOD

The step phenomenon could also be found in creep experiments (Vogel and Hilgner 1979b).

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P.13.2.1.4

Anisotropy of Skin

PURPOSE AND RATIONALE

As is the case with human skin indicated by Langer's lines (Langer 1861; Gibson et al. 1969; Wright 1971; Stark et al. 1977; Daly 1982), the skin of rats exhibits **directional differences** (Hussein 1972, 1973; Vogel and Hilgner 1979a, b; Vogel 1981, 1983a, 1985a, b, 1988; Belkoff and Haut 1991). Stress-strain curves of rat skin showed a different shape if excised perpendicularly or longitudinally to the body axis. Directional variations of mechanical parameters in rat skin were studied depending on maturation and age (Vogel 1981).

PROCEDURE

Male Sprague-Dawley rats are used at different age groups from 1 week up to 24 months. Young animals (1, 2 and 3 weeks) are delivered with their mothers from the breeder. Each age group is randomly divided

into 2 blocks which are assigned as “perpendicular to body axis” or “longitudinal to body axis”. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material of known thickness to measure skin thickness by calipers. From each rat two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out either perpendicular or longitudinal to the body axis, allowing a gauge length between the clamps of an INSTRON instrument of 30 mm. Stress-strain curves are measured at a strain rate of 50 mm/min, whereby the first part of the curve is registered with 10-fold amplification. Besides ultimate values, the extension is measured at given load interval for each curve:

0. 05, 0. 1, 0. 2, 0. 5, 1, 2, 10, 20, and 50 N

and at given stresses:

0. 01, 0. 02, 0. 05, 0. 1, 0. 5, 1, 2, 5, and 10 N/cm².

EVALUATION

Each parameter is measured longitudinal and perpendicular to the body axis. The mean values are statistically compared within each age group using Student's *t*-test.

At low loads, extension is higher in the longitudinal than in the perpendicular direction. The situation is reversed at medium and high stress values. Ultimate extension shows remarkable differences between longitudinal and perpendicular samples. Specimens obtained perpendicular to the body axis showed an increase during maturation, a maximum at 4 months of age and a decrease during further aging. The behavior of samples obtained longitudinal to the body axis was quite different. After an initial rise a maximum was found at 3 weeks. Afterwards a slight decrease was noted. Between 1 and 4 weeks all values of ultimate extension were significantly higher in samples punched out longitudinally to the body axis; between 4 and 12 months they were considerably lower (Vogel 1981).

CRITICAL ASSESSMENT OF THE METHOD

The data indicate the importance of directional variations in all studies of biomechanics of skin.

MODIFICATIONS OF THE METHOD

Directional variations of rat skin were also found in hysteresis experiments (Vogel 1983a) and in relaxation experiments (Vogel 1985a).

Directional variations of the stress-strain curves were also described in the skin of tight-skin mutant mice (Menton et al. 1978).

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P.13.2.1.5

Relaxation Phenomenon

PURPOSE AND RATIONALE

In the relaxation experiment the viscous properties of rat skin are measured (Vogel 1973, 1976a, b, 1983, 1985, 1993a, b).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated subcutaneously

or orally with test drugs or saline. The animals are sacrificed in anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Skin strips are fastened between the clamps of an INSTRON instrument and extended with the high strain rate of 1000 mm/min up to 20% extension. This extension is kept constant for 5 min. The chart speed is initially 1000 mm/min, then 10 mm/min. In this way, the **initial tension** and the **stress values at 0.001, 0.01, 0.1, 1, and 5 min** can be measured. Due to relaxation, the stress values drop down roughly with the logarithm of time.

Furthermore, the **residual stress** after 5 min relaxation period is measured and calculated as percentage of the original stress. After 5 min the sample is returned to 90% of the original strain, for example, from 20% to 18%. The stress following such unloading is recorded and again calculated as percentage of the original stress. Immediately after unloading, the measured stress values rise again spontaneously, which is called **mechanical recovery**. Mechanical recovery is calculated as percentage of initial tension and as percentage of stress after unloading. The relaxation experiment is repeated with increasing degrees of extension of 40%, 60%, 80% and eventually 100% until the specimen breaks.

EVALUATION

For each sample, the relaxation is calculated according to the formula

$$\sigma(t) = A_1 + A_2 \times \log t$$

resulting in two constants (A_1 and A_2) for each sample whereby A_1 is the stress at $t=0$, and A_2 the slope of the relaxation curve.

The **ratio between the constants A_1 and A_2** has to be considered as the most characteristic parameter of the relaxation experiment.

The means of these parameters are compared between treated animals and controls using Student's t -test.

MODIFICATIONS OF THE METHOD

In studies of age dependence in rats skin, a definitive decrease of the **ratio A_2/A_1** was found at 40%

and 60% extension degrees indicating a decrease of plasticity with age. Mechanical recovery, as an indicator of secondary elasticity, was better in old animals at medium extension degrees than in young individuals. Stress relaxation was decreased after corticosteroids and increased after thyroid hormones and D-penicillamine (Vogel 1973, 1993a, b).

Purslow et al. (1998) suggested that relaxation processes within the collagen fibers or at the fiber-matrix interface might be responsible for the viscoelastic behavior of skin.

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P.13.2.1.6

Hysteresis Experiments

PURPOSE AND RATIONALE

In the hysteresis experiment not only the elastic but also the viscous properties of skin are measured (Vogel 1978, 1983).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material of known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens

with a width of 4 mm in the middle of the sample are punched out.

Using the INSTRON instrument, the samples are stretched up to a given extension degree (e. g., 20%) with an extension rate of 20 mm/min. When the given extension is achieved the crosshead is immediately moved back to the starting position with the same velocity. From the upward curve the stress and the modulus of elasticity at the end of the loading phase at the given strain indicating the elastic properties can be measured. When the sample is unstretched the unloading curve shows a different pattern reaching the baseline much earlier than the curve left it during the upward phase. From this point the **residual extension** can be measured. Immediately after the first hysteresis cycle, the experiment is repeated up to an extension degree of 30%, than to 40% and 50% and finally up to 60%.

EVALUATION

By planimetry of the area below the upward curve the **energy input** and of the area between the hysteresis loop the **energy dissipation** can be calculated as well as the **ratio between energy dissipation and energy input** at each hysteresis cycle indicating the viscous properties. Stress and modulus of elasticity at the end of the hysteresis loop, energy input, energy dissipation and the ratio between energy dissipation and energy input at each hysteresis cycle are compared between treated animals and controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

In rat skin, a maximum of the ratio between energy dissipation and energy input was found at 30% to 40% extension. This ratio is influenced by age. At low extension degrees there was an increase with age, whereas at high extension degrees an age-dependent decrease was noted. The ratio dissipation/input was slightly decreased by prednisolone, but definitively increased by D-penicillamine (Vogel 1993b).

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P.13.2.1.7

Isorheological Point

PURPOSE AND RATIONALE

Buss et al. (1976) demonstrated the determination of the isorheological point to be a valuable parameter for the mechanics of connective tissue. This method has been modified and elaborated for the skin strips of rats (Vogel 1984, 1985, 1987).

PROCEDURE

Groups of 20 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated subcutaneously or orally with test drugs or saline. The groups are divided randomly into groups of 10 animals for examination of skin samples either perpendicular or longitudinal to the body axis. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Being fastened between the clamps of an INSTRON instrument, the specimen is expanded rapidly up to 2 N and the corresponding strain is measured. Keeping this strain constant, the load decay (relaxation) is measured for 5 min. Then the sample is again loaded up to 2 N and a second relaxation period of 5 min is evaluated. In the third cycle, the sample is unloaded to 50% of the load observed after the 5-min relaxation period in the second cycle. The phenomenon of **mechanical recovery** is observed. With the crosshead driven up and down, the point is sought where neither immediate relaxation nor mechanical recovery can be observed. The load and strain at this point define the **isorheological point**, which is characterized by the fact that under isometric conditions, the measured load is constant for several minutes. Increasing and decreasing the load by 10% produces a saw-tooth-shaped curve from which the **modulus of elasticity at the isorheological point** can be calculated. The same procedure is performed at higher initial loads such as 10 N and 50 N. The product of percentage of strain multiplied by stress at the isorheological point indicates energy density.

EVALUATION

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

The values showed a decrease during maturation, a minimum at 12 months and an increase during senescence. In studies with desmotropic compounds, the decreased viscosity after treatment with prednisolone acetate was more evident at the isorheological points than at the ultimate values. Likewise, the higher extensibility after treatment with D-penicillamine was indicated more clearly by the isorheological points than by the ultimate strain.

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P.13.2.1.8

Creep Experiments

PURPOSE AND RATIONALE

In creep experiments, viscous behavior of skin is studied. The strain under constant load is also denominated as retardation behavior. (Vogel 1977, 1987).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis three dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

In a special apparatus, skin specimens are suddenly loaded with 100, 200 or 500 g and the extension degree measured. An immediate extension occurs which is followed by a slow and almost continuous creep being measured as **ultimate extension rate**. Furthermore, **extension achieved after 1 h** is registered.

EVALUATION

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

An age-dependent decrease of these parameters was found in rat and human specimens indicating a decrease of viscosity or plasticity with maturation and age. Ultimate extension rate was decreased by prednisolone and increased by D-penicillamine.

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P.13.2.1.9

Repeated Strain

PURPOSE AND RATIONALE

By the method of using repeated strain, mainly the viscous properties of skin are measured (Vogel and Hilgner 1978; Vogel 1987).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

Skin specimens are fastened between the clamps of an INSTRON instrument, extended with a strain rate of 100 mm/min up to 20% extension and immediately unloaded followed by further cycles with the same strain rate and extension degree. The peak of the second cycle is considerably lower than the first, followed by a further decrease in the next cycles. The number of cycles is counted until the stress value is only one half of that of the first cycle. Immediately afterwards, the degree of extension is increased to 30%. Again the number of cycles is counted until the stress value was only one half of that of the first cycle. In this way, the **number of cycles indicating the half-life of tension** due to relaxation is counted at each step of 20%, 30%, 40%, 50%, 50%, 70%, 80%, 90% and eventually 100% extension. The number of cycles decreased from the first step (20%) to the third step (40%) and increased continuously until the last step. This increase was almost an exponential function of the number of steps.

EVALUATION

The means of the number of cycles indicating the half-life of tension due to relaxation at each step are compared between treated animals and controls using Student's *t*-test.

The parameters measured with this method are influenced by several factors: An increase was noted from an age of 1 month up to 24 months. The values were decreased by D-penicillamine and increased by prednisolone treatment. Again this method showed that plasticity of skin is decreased by age and by corticosteroids and increased by D-penicillamine.

MODIFICATIONS OF THE METHOD

Lafrance et al. (1998) tested mechanical properties of human skin equivalents submitted to cyclic forces. The *in vitro* production of disk-shaped (25.4 mm diameter) skin equivalents was based on the culture, under submerged conditions, of keratinocytes seeded on anchorage-based dermal equivalents, a human type I+III collagen gel supplemented with elastin and glycosaminoglycans. The specimens were submitted to quasi-static ramp-deflection cycles induced by means of an actuated hemispherical head. The effects of repeated loading were studied by monitoring the indentation load versus deflection and the relaxation of load over 1000 s.

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P.13.2.1.10

Correlation Between Biomechanical and Biochemical Parameters

PURPOSE AND RATIONALE

Mechanical parameters of skin, such as skin thickness, ultimate load, tensile strength, ultimate strain and ultimate load of elasticity as well as hysteresis, relaxation, isorheological point, creep behavior and values after repeated strain are clearly dependent on maturation and age. They are also influenced by drugs, such as corticosteroids and desmotropic compounds. Therefore, it was of interest to elucidate the correlations of

these parameters with biochemical values such as collagen content, collagen fraction, glycosaminoglycans, and elastin (Vogel 1973, 1976, 1980, 1987, 1988).

PROCEDURE

Mechanical Parameters

Male Sprague Dawley rats are sacrificed under anesthesia; the back skin is shaved and a flap of 5 × 5 cm removed. Subcutaneous fat is removed from the skin flaps. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumbbell-shaped specimens with a width of 4 mm in the middle of the sample are punched out. The specimens are fixed between the clamps of an INSTRON instrument at a gauge length of 30 mm. An extension rate of 5 cm/min is chosen. Tension is recorded at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80 and eventually 90% elongation. The recording starts with high sensitivity and is switched to one-tenth of the sensitivity in order to record the steep end of the curve. Stress-strain curves are determined at an extension rate of 5 cm/min, showing a characteristic shape. During low strain values, there is a gradual increase of load; the curve has a concave part. The stress-strain curve ascends according to an exponential function (Vogel and Hilgner 1977). Afterwards an almost straight part is reached indicating the dependence on Hook's law. At this part the ultimate modulus of elasticity (Young's modulus) can be calculated (increase of load divided by the cross-sectional area). Then some yielding of the curve occurs, which ends in a sudden break of the specimen. This point indicates ultimate strain and ultimate load.

Biochemical Parameters

Total collagen content was measured as mg hydroxyproline/g wet weight of skin according to the method of Stegemann (1958). Collagen was further analyzed according to solubility at 4°C.

| | |
|-------------|------------------------------------|
| Fraction I | soluble in 0.15 mol NaCl solution |
| Fraction II | soluble in 0.5 mol NaCl solution |
| Fraction II | soluble in citrate buffer solution |
| Fraction IV | insoluble. |

Glycosaminoglycan content and the fractions hyaluronic acid, chondroitin sulfates and heparan sulfate were determined by column chromatography. The results were expressed as glucuronic acid per g wet weight. Glucuronic acid was determined according to Bitter and Muir (1960).

Elastin was determined according to Naum and Morgan (1973). The specimen was homogenized in deep-frozen state. A sample of 50 mg was extracted with 0.15 M NaCl solution, later on twice by 5 M guanidine hydrochloride solution at 25°C. After hydrolyzation in an autoclave at 1 atm excess pressure for 45 min, the residue was digested with 0.01% elastase solution (Elastase, Merck, Darmstadt) at 37°C for 2 h. Bovine ligamentum nuchae was used as standard. Elastin was determined in the supernatant as protein according to Lowry et al. (1951). Results were expressed as mg elastin per g wet weight.

EVALUATION

A clear correlation between tensile strength and the content of insoluble collagen was found in all studies for age dependence and the effect of corticosteroids and desmotropic compounds such as D-penicillamine (correlation coefficient 0.9901; Vogel 1974). The values of glycosaminoglycans and of elastin showed age-related dependence and changes after drug treatment, but no significant correlation with the mechanical parameters.

MODIFICATIONS OF THE METHOD

Oxlund et al. (1988) studied the role of elastin in the mechanical properties of rat skin. They concluded that elastin plays a role in the mechanical behavior of rat skin at small stress values and small deformations.

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P.13.2.2

Thermocontraction

PURPOSE AND RATIONALE

If collagenous material such as skin or tendon is heated in a water bath, thermocontraction occurs. This phenomenon was already described by Wöhlisch (Wöhlisch and du Mesnil de Rochemont 1927; Wöhlisch 1932). Verzář and other authors used the phenomenon of thermocontraction of tendons and skin strips extensively to study the ageing process (Verzář 1955, 1957; Lerch 1951; Rasmussen et al. 1964; Boros-Farkas and Everitt 1967; Viidik 1969, 1977, 1979; Vogel 1969). With increasing temperature, a sudden increase of isometric force is found, which is followed by a decrease. To evaluate this phenomenon, either the **shrinkage temperature** or the **stress at and above the shrinkage temperature** can be measured. Furthermore, the decrease of sample strength can be measured.

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. The animals are sacrificed under anesthesia, the back skin is shaved and a flap of 5×5 cm removed. The sample is placed between two pieces of plastic material with known thickness to measure skin thickness by calipers. Perpendicular to the body axis two dumb-bell shaped specimens with a width of 4 mm in the middle of the sample are punched out.

The samples are attached to the clamps of a specifically modified INSTRON instrument which allows the submersion of the test specimen into a beaker filled with 0.9% saline solution. This solution is kept at 58°C or 60°C by a thermostat. Immediately after immersion, the exerted tension is measured and the **maximum of tension** and **time until maximal tension** are recorded.

EVALUATION

The means of these parameters are compared between treated animals and controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Joseph and Bose (1962) tested the shrinkage temperature of skin in newborn rats, at 10 months and 2 years and found an increase from 51.2°C to 61.1°C.

Alain et al. (1977, 1980) tested pieces of dorsal skin of rats. Temperature of maximum tension was decreased from birth to 1 month, and then very slowly increased with age. A rapid relaxation was observed in young rats and in non-senescent adult rats.

Rundgren (1976) found changes of thermal contractility in skin of young and old female rats, and also changes due to repeated pregnancies.

Blackett and Hall (1980) found an increase of thermal shrinkage temperature in two strains of mice during the aging period.

Danielsen (1981) determined thermal stability measured as area shrinkage without tension during heating for membranes of collagen fibrils, reconstituted from solutions of highly purified rat skin collagen.

Allain et al. (1980) built a device, which measured not only hydrothermal shrinking but also swelling in rat skin.

Le Lous et al. (1982a, b, 1983), Flandin et al. (1984) applied the technique of differential scanning calorimetry to evaluate the denaturation process of collagen in rat skin.

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P.13.3**In Vivo Experiments****P.13.3.1****Stress-Strain Curves in Vivo****PURPOSE AND RATIONALE**

In contrast to studies in human beings, animal experiments allow measurement of mechanical properties both *in vivo* under anesthesia and later on *in vitro* (*ex vivo*) at the same site. For this purpose, special methods had to be developed (Barbanel and Payne 1981; Vogel 1981a, b, 1982; Vogel and Denkel 1982, 1985; Denkel 1983).

PROCEDURE

Groups of 10 male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline.

The rats are anesthetized with 60 mg/kg Nembutal i.p. The back skin is shaved mechanically. Skin thick-

ness is measured by use of calipers on an elevated skin fold. Four small (14 × 14 mm) metal plates bearing a hook are used as tabs and are glued on the skin in both longitudinal and perpendicular direction at a distance of 25 mm with a cyanoacrylate preparation. An operation table is mounted on the crosshead of an INSTRON instrument. The table can be turned to allow stretching of the skin in both perpendicular and longitudinal directions relative to the body axis. The anesthetized animals are fastened by their legs to the operation table. A triangle is attached to the load cell. At the end of the triangle, threads are fastened which are conducted by reels and hooked to the tabs. The cross head is moved down manually until the threads are stretched, however, no tension is measured yet. Then the crosshead is driven downwards with a rate of 50 cm/min, what means that the actual extension rate is 100 cm/min. The load is measured only to limited values in order to prevent damage of the skin. In each rat the stress-strain curve is recorded in both directions whereby the order (first longitudinal or first perpendicular) is changed from animal to animal. In this way, the influence of the first extension on the results of the second extension is eliminated. Stress-strain curves are recorded up to an elongation of 80%. Modulus of elasticity is calculated from the almost straight part of the curve.

EVALUATION

Average stress-strain curves with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

In spite of similar conditions, differences between the stress-strain curves from *in vitro* and *in vivo* experiments were found. These findings confirmed the statements of other authors (Barbanel and Payne 1981; Wijn 1980) of the importance of tab distance and tab geometry (Vogel 1981b). Analysis of the data showed that the higher the ratio of distance between the tabs to the area below the tabs, the higher were the stress values. These findings can be explained by the fact that skin consists of several layers. Only the upper layer (epidermis) is fastened to the tabs in the *in vivo* experiment. The forces transmitted to the lower layers are transmitted by a larger area if the area under the tabs is larger. The lower layer can slide over a larger area and is therefore less extended resulting in lower stress values. No sliding is possible if the sample is fastened

from both sides as it is performed in the *in vitro* experiments. Taking into account all experimental conditions including the strain rate, the *in vivo* results are comparable with those obtained *in vitro*. This holds true for the age-dependence as well as for the influence of desmotropic compounds (Vogel and Denkel 1985).

MODIFICATIONS OF THE METHOD

Cook et al. (1977) compared tension/extension ratio curves *in vivo* and *in vitro* in **rats** using the suction-cup method. Baker et al. (1988) described an apparatus for testing mechanical properties of normal and irradiated **pig** skin *in vivo*. In this system, the pads were attached to the skin of the pig rump with double-sided tape. They were moved apart at a predetermined rate using a motorized unit. Force was assessed using a S-shaped, center point, double beam load cell mounted on a movable crosshead. Displacement of the pads was measured using a floating core linear variable displacement transducer. Using this system, Baker et al. (1989) studied the effect of single doses of X-rays on the mechanical properties of pig skin *in vivo*.

Zeng et al. (2001) studied the biorheological characteristics of skin after expansion in **dogs**.

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P.13.3.2

Repeated Strain *in Vivo*

PURPOSE AND RATIONALE

A special method has been developed to study the mechanical properties of rat skin after repeated strain *in vivo* and the course of recovery during different time intervals (Denkel 1983; Vogel and Denkel 1985; Vogel 1988).

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. Groups of 10 animals are used for each time interval tested and for the two directions of testing.

Tabs are fastened on the shaved back skin of anesthetized rats with a distance of 25 mm either perpendicular or longitudinal to the body axis with a cyanoacrylate preparation. With an extension rate of 100 mm/min, the skin is extended 30 times for a 50% strain under anesthesia. Load is recorded and stress calculated by dividing load by skin thickness measured from a skin fold obtained with calipers. The 1st, 5th, 10th, 20th, 25th and 30th cycles are recorded with faster paper speed in order to facilitate the evaluation of modulus of elasticity and stress values. Modulus of elasticity is calculated from the upper part of the stress-strain curve. The area under the curve of 30 cycles is evaluated by computerized calculation according to Simpson's formula (Hütte 1915; Denkel 1983; Vogel 1988).

EVALUATION

Average curves of stress vs. number of cycles with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Stress values decreased after repeated loading approximately with the logarithm of number of cycles. Stress values and the area under the curve were higher perpendicular than longitudinal to the body axis, as found in other *in vivo* experiments (Vogel and Denkel 1985). During the experiment, modulus of elasticity increased

in both directions from the first to the fifth cycle. This may be explained by the so-called "conditioning" of connective tissue (Nemetscheck et al. 1980). From the 5th to the 30th cycle, a decay of modulus of elasticity approximately with the logarithm of number of cycles was noted. The area under the curve calculated from stress values resembled closely the pattern of initial stress, indicating that this value dominates for the area under the curve and that the decay is only of secondary importance.

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P.13.3.3

In Vivo Recovery After Repeated Strain

PURPOSE AND RATIONALE

A method was developed to study the *in vivo* recovery of mechanical properties of rat skin after repeated strain (Denkel 1983; Vogel and Denkel 1985; Vogel 1988, 1990, 1993a, b). Full recovery, i. e., *restitutio ad integrum*, can be observed only by doing *in vivo* experiments but not by *in vitro* experiments.

PROCEDURE

Male Sprague-Dawley rats with an initial weight of 120 ± 10 g are treated for 10 days subcutaneously or orally with test drugs or saline. Groups of 10 animals are used for each time interval tested and for the two directions of testing.

As in the experiment of repeated strain, tabs are fastened on the shaved back skin of anesthetized rats with a distance of 25 mm either perpendicular or longitudinal to the body axis with a cyanoacrylate preparation. With an extension rate of 100 mm/min, the skin is extended 30 times for a 40% strain under anesthesia. Load is recorded and stress calculated by dividing load by skin thickness measured from a skin fold obtained with calipers. The 1st, 5th, and 30th cycles are recorded with faster paper speed in order to facilitate

the evaluation of modulus of elasticity and stress values. Modulus of elasticity is calculated from the upper part of the stress-strain curve. The area under the curve of 30 cycles is evaluated by computerized calculation according to Simpson's formula (Hütte 1915; Denkel 1983; Vogel 1988).

After the first run the animals are returned to their cages with the tabs still in position. A second run of repeated strain is applied after different time intervals at 1, 6, and 16 h. The stress values in the second run are definitively lower than in the first run.

When calculated as percentage of the first run, the differences diminish with extended time intervals. By this *in vivo* method not only the mechanical recovery, which can also be observed *in vitro*, but also the biological recovery, i. e., the restitutio ad integrum, can be measured. Almost full recovery is found after 16 h.

EVALUATION

Average curves of stress vs. number of cycles with standard deviations are plotted for each treatment group perpendicular and longitudinal to the body axis. The stress values and the values for modulus of elasticity of the treated groups are compared with controls using Student's *t*-test. Stress of the first and 30th cycle as well as the area under the curve at the second run are expressed as percentage of the first run after the 1, 6, or 16 h interval. Furthermore, the hours to reach certain percent levels up to 100% are calculated for the stress values during the first cycle and the area under the curve both longitudinally and perpendicularly to the body axis.

CRITICAL ASSESSMENT OF THE METHOD

In the late phase of recovery, an even better restitutio ad integrum was found in animals treated with 300 mg/kg p.o. D-penicillamine or 10 mg/kg s.c. prednisolone acetate. In contrast to other biomechanical parameters, the restoration process was found to be barely influenced by treatment with desmotropic compounds.

Surprisingly, in studies on age dependence restitutio ad integrum was the fastest in old animals. The ability of the dermis to reconstitute the fibrous structure is apparently not influenced negatively by age.

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P.13.4

Healing of Skin Wounds

PURPOSE AND RATIONALE

Healing of skin wounds is a multiphasic process. The effect of drugs on the healing process was studied by measuring the mechanical strength at various time intervals after incision of the skin (Vogel 1970).

PROCEDURE

Groups of 10 to 20 male Sprague-Dawley rats weighing 120 ± 5 g are used for each dosage or control and for each test interval. Under anesthesia the dorsal skin is shaved and an approximately 3 cm long incision is made down to the fascia in a cranio-caudal direction in the dorso-lumbar region. Immediately afterwards, the wound is closed with wound clips. The rats are treated subcutaneously with test drugs beginning on the day of surgery. The clips are removed the day before the tensile strength is tested or on the 10th post-operative day at the latest.

For the measurement of wound tensile strength, the rats are sacrificed under anesthesia on days 3, 6, 9, or 12 after surgery. Wound clips are fastened *in situ* on each side of the incision and connected by means of threads with the load cell and the crosshead of an INSTRON-Instrument. Stress-strain curves are recorded at an extension rate of 5 cm/min. **Dehiscence of the wound** results in a sudden drop of the registered load. For experiments up to 3 weeks, bell-shaped skin strips are punched and tensile strength is tested, as described for evaluation of tensile strength in normal skin.

EVALUATION

Mean values of tensile strength of skin wounds in drug treated groups at each time interval are compared with controls using Student's *t*-test. To visualize the influ-

ence of drugs on the healing process, the changes following treatment with drugs are expressed as percentage of vehicle treated controls.

A dose-dependent decrease following treatment with corticosteroids is found after immediate postoperative treatment and a dose-dependent increase in prolonged experiments. This data serve as comparative parameters for new test compounds

MODIFICATIONS OF THE METHOD

Many authors measured tensile strength of skin wound to follow the course of wound healing under various conditions. Most experiments were performed in **rats** (Struck et al. 1967; Corps 1969; Holm-Pedersen and Zederfeldt 1971; Holm-Pedersen and Viidik 1972a, b; Andreassen et al. 1977; Andreassen and Oxlund 1987; Greenwald et al. 1993; Seyer-Hansen et al. 1993; Jung et al. 1994; Adamson et al. 1996; Brunius and Ahren 1996; Oxlund et al. 1996; Paul et al. 1997; Taylor et al. 1997; Quirinia and Viidik 1991, 1998; Canturk et al. 1999; Gupta et al. 1999), with some of them also using the INSTRON-Instrument (Phillips et al. 1993; Maxwell et al. 1998; Jimenez and Rampy 1999; Kim and Pomeranz 1999).

A biphasic effect of corticosteroids on wound healing in rats was also found by Oxlund et al. (1979).

Forslund et al. (2006) published a comparative dose-response study of cartilage-derived morphogenetic protein (CDMP)-1, -2 and -3 for tendon healing in rats.

Furthermore, **mice** (Butler et al. 1991; Celebi et al. 1994; Kashyap et al. 1995; Vegesna et al. 1995; Gonul et al. 1998; Matsuda et al. 1998), **guinea pigs** (Bernstein et al. 1991; Drucker et al. 1998; Silverstein and Landsman 1999), **rabbits** (Sandblom 1957; Wu and Mustoe 1995; Pandit et al. 1998, 1999; Knabl et al. 1999; Xia et al. 1999), **dogs** (Howes et al. 1929; Al Sadi and Gourley 1977; Scardino et al. 1999), **pigs** (Langrana et al. 1983; Higashiyama et al. 1992; Chang et al. 1998; Fung et al. 1999) or **Yukatan miniature pigs** (Van Dorp et al. 1998) were used.

Using PPAR α , β and γ mutant mice, Michalik et al. (2001) demonstrated that PPAR α and β are important for the rapid epithelialization of a skin wound and that each of them plays a specific role in this process. PPAR α is mainly involved in the early inflammatory phase of the healing, whereas PPAR β is implicated in the control of keratinocyte proliferation.

PPAR β is discussed as a target for wound healing drugs (Tan et al. 2003).

Smith et al. (2001a, 2001b) discussed the role of peroxisomes in dermatology.

In some studies simultaneously polyvinyl alcohol sponges were implanted in which collagen accumulation was determined (Albina et al. 1993; Schaffer et al. 1996; Koshizuka et al. 1997; Bitar 1998; DaCosta et al. 1998; Witte et al. 1998).

Ågren and Mertz (1994) found excessive granulation tissue formation and retarded wound contraction in wounds in tight-skin mice.

Kyriakides et al. (1999) reported accelerated wound healing in mice with a disruption of the thrombospondin 2 gene.

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P.14

Protection Against UV Light

PURPOSE AND RATIONALE

The mechanical parameters' skin thickness, ultimate strain, ultimate load, tensile strength and modulus of elasticity were used to evaluate the effects of UV-irradiation and the prevention of skin damage in hairless mice (Alpermann and Vogel 1978; Vogel et al. 1981).

PROCEDURE

Female hairless mice (strain mutant h/h) with an initial weight of 20 ± 2 g are used. Groups of 20 animals are treated topically with 0.025 ml of the sunscreen product or the base. One other group is not treated topically, but irradiated. A further group is neither treated nor exposed to UV-B.

A special light source (Osram Ultra-Vitalux No. 2) is used. This light source is almost free from UV-C. For UV exposure groups of 5, mice are immobilized under a fine wire net of 14×14 cm at a distance of from the lamp, resulting in an irradiation energy of 14 mW/cm^2 for UV-B and 33 mW/cm^2 for UV-A. Irradiation is per-

formed once a day except Saturday and Sunday during 4 weeks. Exposure time is 45 s in the first week, 60 s in the second, 90 s in the third, and 120 s in the fourth week. The animals are sacrificed under anesthesia 72 h after the last irradiation. A flap of back skin is removed, skin thickness measured by use of calipers and two dumb-bell shaped samples punched with a width of 4 mm in the middle of the specimen. Stress-strain curves are recorded with an INSTRON-instrument at a strain rate of 5 cm/min. Ultimate load and ultimate strain are recorded and tensile strength as well as ultimate modulus of elasticity are calculated.

Furthermore, samples of skin are deep-frozen for chemical analysis of collagen and soluble collagen fractions as well as of elastin (Vogel 1978).

EVALUATION

The mean data from animals treated with sunscreen products are compared with those of animals treated with ointment base, irradiated and non irradiated controls using ANOVA and Student's *t*-test.

MODIFICATIONS OF THE METHOD

The effect of UV-irradiation on skin has been studied by several authors both in man and in animals.

Wolska (1974) recommended the hairless mouse as an experimental model for evaluating the effectiveness of sunscreen preparations.

Cook et al. (1979) investigated the changes in the mechanical properties of intact guinea pig skin resulting from ultra-violet irradiation.

Lowe and Breeding (1986) evaluated several sunscreen preparations by UVB irradiation of mice. Sunscreen solutions were applied to the back of 5–8 weeks old female skh/HR-1 mice one h before irradiation with FS40 sunlamps. Epidermal DNA synthesis assay was used to measure sunscreen efficacy. The amount of UVB required to achieve a 50% suppression of radiolabelled thymidine incorporation in treated and untreated mice was compared as a ratio to determine the protective factor. Furthermore, epidermal ornithine decarboxylase activity was determined by measuring the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine 24 h after a single UVB exposure. Edema was estimated by the increase in double skin fold thickness measured by a caliper at 24 h post irradiation.

Benrath et al. (1995) reported that substance P and nitric oxide mediate wound healing of ultraviolet photodamaged skin in rats.

Muizzudim et al. (1998) studied the effect of topical application of antioxidants and free radical scavengers on protection of hairless mouse skin, exposed to

suberythral doses of ultraviolet B three times a week, and measured epidermal thickness by microscopy.

Fullerton and Keiding (1997) quantified UV-B induced erythema in depilated Hartley-strain albino male guinea pigs and compared the results with a tristimulus colorimeter (Minolta ChromaMeter CR-200) and two spectrophotometers (Minolta Spectrophotometer CM-508i and CM-2002). With the tristimulus colorimeter the color is expressed in a three-dimensional color space, which simulates the perception of color by the human eye.

Nishimori et al. (2001) described degenerative alterations of dermal collagen fiber bundles in photodamaged human skin and UV-irradiated hairless mouse skin and the effects on decreasing skin mechanical properties and appearance of wrinkles.

Oba and Edwards (2006) studied the relationships between changes in mechanical parameters of the skin, wrinkling, and destruction of dermal fiber bundles caused by photoaging on hairless mice irradiated with UVB light.

Further description of ultraviolet erythema in animals see Sect. H. 3.2.2.1.

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P.15

Transepidermal Water Loss (TEWL)

PURPOSE AND RATIONALE

The physical basis for the measurement of transepidermal water loss (TEWL) is the diffusion law discovered by A. Fick in 1855.

$$dm/dt = -D \times A \times dp/dx$$

where:

A = surface (m^2),

m = water transported (g),

t = time (h),

D = diffusion constant ($=0.0877 \text{ g/m} \times h \times \text{mm Hg}$),

p = vapor pressure of the atmosphere (mm Hg),

x = distance from skin surface to point of measurement.

Most of the recent studies are performed with commercially available instruments, such as Tewameter TM 210, Courage and Khazaka, Cologne, Germany, Evaporimeter EP1, Servo Med AB, Vallingby, Sweden, primarily designed for the used in human beings.

Several studies were performed in **rats**, e. g., in hairless rats (Doucet et al. 1991; Vanbever et al. 1998), neonatal rats (Wickett et al. 1995), in rats with experimentally induced condition of essential fatty acid deficiency (Basnayake and Sinclair 1956; Prottey et al. 1976; Hartop et al. 1976, 1978; Penneys 1992; Yamaguchi et al. 1998; Meguro et al. 2000), **hairless mice** (Grubauer et al. 1989; Mortz et al. 1997; Sato et al. 1998), essential fatty acid deficient hairless mice (Menton 1968; Lowe and Stoughton 1977), platelet-type 12-lipoxygenase deficient mice (Johnson et al. 1999), keratin 10 deficient mice (Jensen et al. 2000), **guinea pigs** (Frosch et al. 1993; Fuchs et al. 1998; Sagiv et al. 2000), **pigs** (Zhao and Singh (1999) **Yucatan micro-swine** (Gendimenico et al. 1995).

Löffler et al. (2001) evaluated irritant skin reaction in mice by measurement of auricular transepithelial water loss.

PROCEDURE

BALB/c mice aged 10–16 weeks are sensitized on day 0 by applying 50 µl 2,4-dinitro-fluorobenzene (DNFB) solution (0.5% diluted in acetone/olive oil 4:1) to the shaved dorsal neck region. On day 5, the dorsal surface of one ear is challenged by applying 10 µl DNFB 0.3%; the other side is treated by acetone/olive oil alone.

Transepithelial water loss is measured with an evaporimeter (Tewameter TM 210, Courage and Khazaka, Cologne, Germany) with a measuring cylinder into which the whole ear of the mouse can be placed. The measurements are performed under isofluoran inhalation anesthesia. Measurements are performed before and 24 h after challenge.

EVALUATION

The comparison between the treated and untreated ears is calculated by the Wilcoxon test. The comparison between the tested groups is calculated by the Kruskal-Wallis *H*-test, after the Mann-Whitney *U*-test.

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P.16 Skin Hydration

PURPOSE AND RATIONALE

Techniques to characterize the barrier function of the skin include a number of noninvasive methods to measure moisture content and loss through the skin surface. One of these measurements is the determination of skin hydration using a method known as corneometry (Bilchmann and Serup 1988). This technique determines the capacitance of the skin due to its behavior as a dielectric medium and assesses a 10- to

20- μ m thickness of the stratum corneum. The method has been compared with other techniques (Van Neste 1991; Fluhr et al. 2001) and applied to evaluate dermatological and cosmetic products in human volunteers (Zuang et al. 1997; Singh et al. 2001; Yilmaz and Borchert 2006).

Jensen et al. (2000) studied skin hydration in keratin-10-deficient mice. Kappes et al. (2004) used this method to investigate the quality of human xenografts on SCID mice. Hester et al. (2004) evaluated this method in dogs.

PROCEDURE

Normal adult dogs (female Beagle and male hound-cross dogs) were used. Hair was clipped from the left inguinal region of each dog 1 week before measurements to minimize any effects of recent hair clipping. Because hair grows sparsely in the inguinal region, only a minimal amount of clipping was necessary. To measure skin hydration, a Corneometer 825 meter (Courage Khazaka Electronics) was used. Animals were gently restrained in a right-lateral recumbency on a padded floor and were given time to relax so that movements could be minimized during data collection. The probes were held in place manually, and readings were taken for 20 s each. The data were stored electronically using a laptop computer and appropriate software. Values representing an average of at least ten determinations were used to calculate average values.

EVALUATION

Statistical analyses were conducted by repeated-measures ANOVA for main time-and-breed effects and time \times breed interactions with Tukey' multiple comparisons performed at *P* values <0.05.

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P.17

Influence on Hair Growth

PURPOSE AND RATIONALE

Several mutations affecting hair growth or quality have been reported in the laboratory mouse (Holland 1988), among them “alopecia” (Dicke 1955); “alopecia periodica” (Tutikawa 1952); “crinkled” (Falconer et al. 1951); “frizzy” (Falconer and Snell 1952); “fuzzy” (Mann 1964); “rhino” (Mann 1971); “naked” (Raphael et al. 1982); “nude” (Flenagen 1966; Buhl et al. 1990; Militzer 2001); “ragged” (Slee 1962); androchronogenetic alopecia (AGA) mouse (Matias et al. 1989); aging C3H/HeJ mice (Sundberg et al. 1994); SPF-ASH mice (Shimada et al. 1994); “nackt” (Benavides et al. 1998); transgenic mice overexpressing homeobox gene *MSX-2* (Jiang et al. 1999). Kligman (1988, 1998) used the *Skh*-hairless mouse as a model for evaluating promoters of hair growth.

PROCEDURE

Six litters of *Skh*-hairless-1 albino mice with 5–8 animals are housed, with their mothers, in individual plastic cages. Food and water are supplied ad libitum. Beginning at 1 week of age, topical treatment is applied: two litters dorsal application of low dose (e.g., 0.2% minoxidil), two litters dorsal application of high dose, two litters abdominal application of high dose. Application is performed once daily five times a week at a rate of 20 μ l/cm² of skin using a digital micropipette. Two control litters receive no treatment. All treatment continue for up to 17 weeks, after which mice are sacrificed and grossly examined for signs of toxicity.

Mice are observed daily for hair growth and weighed each week. Photographs are taken when hair growth appears to be maximum. At the time of maxi-

mal hair growth, two or three representative neonates from the treated and untreated groups are sacrificed for skin biopsies; all remaining mice continue on treatment to assess the extent of a third pelage. Biopsy specimens are fixed in formalin and stained with hematoxylin and eosin.

EVALUATION

In transverse sections, all active hair matrices or viable follicles, which contain keratinized hairs located in the subcutis, are counted. Fourteen to 16 contiguous fields are examined over a surface distance of 1 cm at 250× magnification. Mean values of the groups are compared using Student's *t*-test.

MODIFICATIONS OF THE METHOD

De Brouwer et al. (1997) studied the effects of a non-steroidal anti-androgen on human hair production by balding scalp grafts maintained in testosterone-conditioned **nude mice**. Samples of balding human scalp were grafted on to the left flank of nude mice (bal/c, nu/nu). All mice received a topical application of 300 µg/10 µl testosterone propionate on the non-grafted flank. Testosterone conditioning started 5–7 weeks after grafting and went on until the end of the experiment, once daily 5 days a week. Anti-androgen/placebo treatment was started before hair production was visible. Hair production potential was assessed according to the number and diameter of the hairs.

A hair loss mutation on mouse chromosome 19, called **scraggly**, was described by Herron et al. (1999).

Several authors studied **chemotherapy-induced alopecia in mice, rats and rabbits** (Powis and Kooistra 1987; Paus et al. 1994a, b; Cece et al. 1996; Sredni et al. 1996).

The **Dundee experimental bald rat (DEBR)** was used as model for alopecia areata (Oliver and Lowe 1995; McElwee et al. 1997).

Kimura (1996) underlined the usefulness of studies in **hairless descendants of Mexican hairless dogs** in dermatological science.

The **balding stumptail macaque** is recommended as a model for androgenetic alopecia (Brigham et al. 1988; Diani et al. 1995; Pan et al. 1998).

The stimulating effect of drugs on hair growth has been studied using *in vitro methods*.

Kurata et al. (1996) investigated the effect of hypertrichotic agents on follicular from macaque and human skin and nonfollicular cells (normal keratinocytes and dermal fibroblasts) *in vitro*. Minoxidil induced a significant increase in all follicular cells in a dose-

specific manner, whereas nonfollicular cells showed no response.

Lachgar et al. (1996) found inhibitory effects of bFGF, VEGF and minoxidil on collagen synthesis by cultured hair dermal papilla cells from rat vibrissa follicles.

Boyera et al. (1997) described biphasic effects of minoxidil on the proliferation and differentiation of normal human keratinocytes obtained from microdissected hairs of from plucked hairs. Minoxidil stimulated human keratinocyte proliferation at micromolar doses, while antiproliferative, pro-differentiative and partially cytotoxic effects were observed with millimolar concentrations.

Sato et al. (1999) reported that minoxidil increases 17β-hydroxysteroid dehydrogenase and 5α-reductase of cultured human dermal papilla cells from balding scalp.

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P.18 Cutaneous Microcirculation

P.18.1

General Considerations

PURPOSE AND RATIONALE

Various techniques are used to determine cutaneous blood flow, such as radioactive microspheres, xenon clearances, plethysmography, laser Doppler velocimetry (Flagrell 1986; Guy et al. 1985), reflectance spectrophotometry (Kimura et al. 1988; Kakizoe et al. 1992).

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P.18.2

Laser Doppler Velocimetry

PURPOSE AND RATIONALE

Laser Doppler velocimetry and laser Doppler flowmetry are essentially identical procedures. With these methods, light is transmitted from a helium-neon laser source in the instrument to the skin via an optical fiber. The laser provides light of single frequency (wave-

length 632.9 nm) and allows the Doppler effect to be exploited. The incident radiation enters the skin tissue and is multiply scattered and reflected by non-moving components and by the mobile red blood cells that are encountered as the radiation penetrates to a depth of 1–1.5 mm. A portion of scattered/reflected incident radiation exits the skin and is collected by second or third optical fibers that carry the light back to the instrument. The returning radiation falls on a photodetector and is converted to an electrical signal. Stationary skin tissue reflects and backscatters light at the same frequency as the incident source. Erythrocytes moving with certain velocity, however, reflect radiation that is slightly frequency-shifted, the shift increasing with increasing velocity.

Hirkaler and Rosenberger (1989) described simultaneous two-probe laser Doppler velocimetric assessment of topically applied drugs as a simple, non-invasive method for the determination of cutaneous blood flow in anesthetized rats.

PROCEDURE

Male Sprague-Dawley rats (400–500 g) are anesthetized with urethane (1.6 g/kg), the abdominal region carefully clipped and the remaining hair removed with a commercially available depilatory. Phthalate buffer (pH 4.0) is gently applied to neutralize the effects of the depilatory cream. The rat is placed on its back and allowed to stabilize for approximately 15 min. Cutaneous blood flow is measured using two standard Medpacific LD 5000 capillary perfusion monitors and probes (1.9 cm diameter). The probes are modified to allow application of the drug without removal of the probe. This is achieved by enlarging the center opening of the adhesive pad to 1 cm, which creates a drug well of approximately 5 μ l vol. The drug is introduced through a short length of polyethylene tubing (PE10) held in place by the adhesive pad and placed slightly off center to avoid interference with the sensing device.

The modified probes are attached to the lower abdominal region of the rat, approximately 1–2 cm from the midline. Cutaneous blood flow is recorded continuously from 30 min prior to drug application and for 5 h post-dosing. Various doses of standard (0.015 to 0.75 mg/kg minoxidil) or test compound or control vehicle (propylene glycol 15%, ethyl alcohol 65%, water 20%) are applied. At the end of the experiment, the animals are sacrificed.

EVALUATION

Post-dosing values are expressed as % change from control and analyzed using Student's paired *t*-test.

MODIFICATIONS OF THE METHOD

Knight et al. (1987) measured microvascular blood flow by a laser Doppler flow meter in **rabbit** epigastric island flaps made ischemic for various intervals of time.

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P.18.3

Measurement of Skin Microcirculation by Reflectance Spectroscopy

PURPOSE AND RATIONALE

Kimura et al. (1988), Kakizoe (1992) developed reflectance spectrophotometric measurement to analyze the microcirculation of the skin in real time. The light reflected from the skin tissue containing information about hemoglobin, the injected dye, and other data, is continuously analyzed into the relative absorption spectrum and the changes in relative absorption values at specific wavelengths are used as indices of oxyhemoglobin content and capillary permeation.

PROCEDURE

Male Wistar rats weighing 300–400 g are anesthetized with pentobarbital (40 mg/kg i.p.). The skin of the back is shaved and the hair removed by a commercial hair remover. A polyethylene catheter (PE10) is inserted into the femoral artery to measure blood pressure on a polygraph. An other catheter is inserted into the femoral vein for injection of drugs or Evans blue.

To obtain the relative absorption spectra, a spectro-multi-channel-photodetector system (MCPD 110; Otsuka Electronics Co., Ltd., Osaka, Japan) is used. White light (halogen lamp 150 W) is projected onto the skin through an optical fiber. The light reflected from the skin is transmitted to the detector system via another optical fiber. The positions of the optical fibers are fixed independently. Each fiber is attached to a steel arm on the stand, and the height of the arms is adjusted so that the top of the fibers can be in gentle contact with surface of the skin. The angles of the fibers are adjusted so that the relative absorbance spectrum with the best peaks of oxyhemoglobin can be obtained. The reflected light is passed through a slit and illuminated

on the grating surface to obtain the spectrum. Following amplification by an image intensifier, the component wavelengths are sampled by photodiode array in a short time interval. The relative absorbance spectra from the skin against the spectra for the white light are obtained at wavelengths ranging from 450 to 643 nm, arranging the initial relative absorbance at 640 nm to zero. The sampling time is 50 ms. The average of 10 measurements is indicated at 1-s intervals on the output unit. The spectrum at a point of time and time-dependent changes in relative absorbance values at selected wavelength are shown on the display and registered by an X-Y recorder.

After achieving a steady state under anesthesia, the relative absorbance spectrum is obtained from the skin, and two peaks at wavelength of about 540 and 577 nm, corresponding to those of oxyhemoglobin, are observed. The relative absorbance spectrum is measured at the posterior part of the back near the backbone because the shape is relatively flat in this region and it is easy to fix the optical fibers.

To standardize the changes in oxyhemoglobin content in skin tissue, graduated doses of noradrenaline are injected intravenously and the effect on relative absorbance is measured.

To measure the content of oxyhemoglobin and permeation of the capillaries at the same time, 1.5 ml/kg 0.05% Evans blue solution is injected intravenously 10 min before measurement. Histamine (0.3–100 µg/50 µl/site) is injected intradermally into the skin of the back, and measurement is started 1 min after the injection. The changes in the relative absorbance values at 540 nm, an absorption peak in the oxyhemoglobin spectrum, and at 610 nm, an absorption peak in the Evans blue spectrum, are measured for 15 min, and the absorption values at baseline and the point of maximum change are compared.

EVALUATION

Results are expressed as mean \pm SEM. Comparisons are made using the Student's *t*-test or Mann-Whitney's *U*-test.

MODIFICATIONS OF THE METHOD

Hertel (1986, 1992) measured cutaneous microcirculation in the pinnal of conscious rats. Erythrocyte flow velocities were measured by the 'flying spot technique' (Tyml and Ellis 1982) and the diameters of the capillaries were measured from a monitor with a ruler.

Da Costa et al. (1992) measured the fluctuations in the diameter of selected arterioles in the cutaneous microcirculation of **Syrian golden hamster** dorsal skin

flap chambers. These ranged in size between 10 and 70 µm at different branching order sites, before burn, at the same site after burn and after injection of drugs.

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P.19

Isolated Perfused Skin Flap

PURPOSE AND RATIONALE

Riviere et al. (1986) and Riviere and Monteiro-Riviere (1991) described the isolated perfused porcine skin flap as an *in vitro* model for percutaneous absorption and cutaneous toxicology studies. This group has used the model for various purposes (Riviere et al. 1995; Vaden et al. 1996; Baynes et al. 1997; Inman et al. 2003; Monteiro–Riviere et al. 2003).

PROCEDURE

Weanling female Yorkshire pigs weighing 20–30 kg are acclimated for 1 week prior to the study. The pigs are housed on elevated floors and provided water and 15% protein pig and sow pellets ad libitum.

The isolated perfused porcine skin flap is a single, axial pattern tubed skin flap obtained from the ventral abdomen of female weanling swine. Two flaps per pig, each lateral of the ventral midline, may be obtained in a single surgical procedure. The surgery involves two steps: creation of the flap in stage I and harvest in stage II. Pigs weighing approximately 20–30 kg are pre-medicated with atropine sulfate and xylazine hydrochloride, induced with ketamine hydrochloride, and inhalation anesthesia is maintained with halothane. Each pig is prepared for routine

surgery in the caudal abdominal and inguinal region and a 4 × 12 cm area of skin is demarcated. Following incision and scalpel dissection of the subcutaneous tissue, the caudal incision is apposed, sutured, and the tubed skin flap edges trimmed of fat and closed. Two days later, a second surgical procedure is used to cannulate the artery and harvest each of these skin flaps. The isolated perfused porcine skin flap is then transferred to a custom-designed temperature- and humidity-regulated perfusion chamber. A computer monitors perfusion pressure, flow, pH, and temperature. The flaps are perfused for 1 h prior to dosing with a modified Krebs/Ringer bicarbonate buffer (pH 7.4) containing bovine serum albumin, glucose, penicillin G amikacin, and heparan, during which 1.0-ml arterial and 3.0-ml venous samples are collected to assess glucose utilization. Once flap viability is confirmed, the perfusion is interrupted and each flap is removed from the chamber. Flap perfusion is resumed, with the venous perfusate sampled every 15 min for the first 2 h and every 30 min thereafter for the 8-h duration. Absorption is defined as the total amount of radioactivity detected in the perfusate over an 8-h perfusion period and expressed in units of % of applied radioactivity or mass absorbed. Penetration is defined as the sum of total radioactivity found in the perfusate (absorption), stratum corneum, fat and dosed skin samples. Penetration is the maximum amount of test compound that could be absorbed into the systemic circulation if all test compounds within the skin ultimately were absorbed over a prolonged period of time. Penetration is also relevant to the direct toxic effect within the skin.

EVALUATION

Total recovery is calculated as the sum of penetration plus the amount of radioactivity found in the washing samples. All data are expressed as mean ± standard error of the mean (SEM). The significant differences ($P < 0.05$) across each set of treatments are determined by ANOVA in PC SAS (SAS Institute, Cary, N.C., USA; version 8.01). Significant differences ($P < 0.05$) between each mean and paired nonaqueous counterpart are determined using a Student's *t*-test.

MODIFICATIONS OF THE METHOD

Pickens et al. (1994) studied the effect of extended perioperative pentoxifylline on random skin flap survival in Yorkshire pigs.

Skin flap models have been described for several animal species, mostly in **rats** (Hahn et al. 1993; Stadelmann et al. 1998; Yenidunya et al. 1998; Cottler et al. 1999; Yang and Morris 1999; Jones et al. 2001; Ok-

sar et al. 2001; Lay et al. 2003; Mittermayer et al. 2003; Ozkan et al. 2004; Agaoglu and Siemionow 2005; Ulusal et al. 2005; Hosnuter et al. 2006). Lineaweaver et al. (2004) described the influence of vascular endothelium growth factor and surgical delay on survival of the transverse rectus abdominis myocutaneous flap in rats. The model is based on the right abdominis rectus muscle as carrier and inferior epigastric vessels as vascular pedicle. Following induction of general anesthesia, the abdominal regions were shaved, and a rectangle measuring 3 × 8 cm was drawn onto the upper abdominal area of the rats. The borders of the skin paddle of the proposed transverse rectus abdominis myocutaneous flap were cut down to deep fascia. The branches of both superficial epigastric vessels joining the flap were ligated and cut. Both rectus abdominis muscles were divided at the superior border of the skin paddle. The superior deep epigastric vessels of both rectus abdominis muscles were divided. The skin incision was then closed using 4–0 nylon sutures.

Barker et al. (1989) and Minh et al. (2002) described skin flap models in **mice**.

Degner et al. (1996) described a medial saphenous fasciocutaneous free flap in **dogs**.

Teunissen et al. (2004) evaluated the primary critical ischemia time for the deep circumflex iliac cutaneous flap in **cats**.

Bristol et al. (1991) reported preparation and metabolic parameters of an isolated perfused skin flap in **horses**.

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Safety Assays in Skin Pharmacology

For safety assays in skin pharmacology, see Maibach (2006).

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Chapter Q

Guidelines for the Care and Use of Laboratory Animals¹

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Q.1 Regulations for the Care and Use of Laboratory Animals in Various Countries

See Table Q.1 on the following page.

Q.2 Techniques of Blood Collection in Laboratory Animals

Q.2.1 Introduction

Blood is collected from laboratory animals for various scientific purposes, for example, to study the effects of a test drug on various constituents, such as hormones, substrates, or blood cells. In the field of pharmacokinetics and drug metabolism, blood samples are necessary for analytical determination of the drug and its metabolites. Blood is also needed for some *in vitro* assays using blood cells or defined plasma protein fractions.

The techniques for blood collection depend on specific factors which differ from one experiment to the other. There is a difference between terminal and non-terminal blood collection techniques. The conditions of blood collection at the end of an experiment which includes death of the animal (terminal experiment) are completely different (anesthesia, volume of blood)

¹By A.W. Herling, J. Maas, K. Seeger (1st edition), revised by U. Albus (2nd and 3rd edition).

Table 1 Regulations for the care and use of laboratory animals

| Country, Institutions | Legislation |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Australia National Health and Medical Research Council Commonwealth Scientific and Industrial Research Organisation Australian Agricultural Council | Australia is governed by a national Government, six State Governments, and two Territory Governments. Animal welfare within Australia is a State/Territory responsibility. The Australian Animal Welfare Strategy (AAWS) from 2005 http://www.daff.gov.au/ http://www.animallaw.info/ |
| Austria Ministry of Agriculture | Bundesgesetz über den Schutz der Tiere (TSchG), BGBl. I Nr. 118/2004 http://www.vu-wien.ac.at/vetrecht/TSchG%20BGBl.pdf |
| Belgium | In 1991 the provisions of the ETS 123 convention were enacted in the Belgian legislation through the “Law of 18 October 1991 approving the European Convention on protection of vertebrate animals used for Experimental or other Scientific Purposes and its Annexes A and B done at Strasbourg on 18 March 1986”. In 1994 the “Royal Decree of 14 November 1993 related to the protection of experimental animals” was enacted. |
| Canada | Canadian Council on Animal Care http://www.animallaw.info |
| China (Peoples Republic of China) State Science and Technology Commission, Beijing | Regulations for the Administration of Affairs Concerning Experimental Animals, Nov. 1988 Implementing Regulations of the Administration on Medical Experiments on Animals, June 1989 http://www.nal.usda.gov/awic/legislat/internat.htm http://www.animallaw.info/ |
| Denmark | LBK (Lovbekendtgørelse) nr. 726 af 9. sept. 1993: Bekendtgørelse om lov om dyreforsøg A minor change has been added to the above about animals that are released in connection to an experiment: <ul style="list-style-type: none"> • LOV nr. 386. 6 juni 1991 (animal protection law) • LOV nr. 1081 af 20. dec. 1995: Lov om ændring af lov om dyreforsøg • BEK (Bekendtgørelse) nr. 332 af 18. maj 1990: Bekendtgørelse om fremskaffelse af dyr til forsøg • BEK nr. 27 af 22. jan. 1996: Bekendtgørelse om ændring af bekendtgørelse om fremskaffelse af dyr til forsøg • BEK nr. 716 af 1. aug. 1994: Bekendtgørelse om forretningsorden for Rådet for Dyreforsøg • BEK nr. 333 af 18. maj 1990: Bekendtgørelse om forsøgdysr pasning og opstaldning af om udryddelsestruede og vildtlevende dyr til forsøg m.v. • BEK nr. 739 af 6. dec. 1988: Bekendtgørelse over og indberetning om dyreforsøg Legislation revised in 1993 in conformity with EU directives http://www.retsinfo.dk/ |
| European Union | Council of Minister’s Directive 86/609/EEC (1986) on the Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and other Scientific Purposes http://europa.eu.int/ European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. December 15, 1990 http://www.uku.fi/laitokset/vkek/Sopimus/convention.html |
| Federal Republic of Germany Ministry for Food, Agriculture and Forestry | Neufassung des Tierschutzgesetzes vom 18. Mai 2006 Richtlinie vom 24. November 1986 zur Annäherung der Rechts- und Verwaltungsvorschriften der Mitgliedstaaten zum Schutz der für Versuche und andere wissenschaftliche Zwecke verwendeten Tiere (86/609/EWG) Gesetz zu dem Europäischen Übereinkommen vom 18. März 1986 zum Schutz der für Versuche und andere wissenschaftliche Zwecke verwendeten Wirbeltiere vom 11. Dezember 1990 http://www.bml.de/ and http://www.uni-giessen.de/tierschutz/ |

Table 1 (continued)

| Country, Institutions | Legislation |
|------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Finland | Act on Animal Protection (247/1996, amend. 1194/1996 and 594/1998), Statute on Animal Protection (396/1996, amend. 402/1998), Statute on the Transport of Animals (491/1996, amend. 1398/1997 and 955/1998) and Statute on Animal Experimentation (1076/1985, amend. 395/1996) http://www.nca-nl.org/ |
| France | No uniform national animal welfare law, but several decrees. “Loi à la protection de la nature”, 10 juillet 1976 “Décret aux expériences pratiquées sur les animaux” 19 octobre 1987 Arrêtés interministériels (3 arrêtés), 19 avril 1988 http://www.nca-nl.org/ |
| Italy | Legislative Decree no. 116 of January 27, 1992, enforcing European Recommendations contained in Directive 86/609/EEC Specific Law of October 12, 1993 http://www.nca-nl.org/ |
| Ireland Department of Agriculture and Food | Cruelty to Animals Act, 1876 European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994 http://www.agriculture.gov.ie/ |
| Japan Prime Minister author- ized to set standards for minimum pain provision | Law Concerning the Protection and Control of Animals (Law No. 105, October 1, 1973, revised June 15, 2005) http://www.alive-net.net/english/en-law/L3-outline.html |
| New Zealand Animal Welfare Advisory Committee (AWAC), es- tablished in 1989 by the Minister of Agriculture | Animal Welfare act 1999, Commenced January 1, 2000 http://rangi.knowledge-basket.co.nz/gpacts/public/text/1999/an/142.html The codes of recommendations and minimum standards for the welfare of animals, endorsed as a national code on 25 May 1995 http://www.maf.govt.nz/mafnet/profile/legislation/ |
| Netherlands Minister of Health, Welfare and Sport | Dutch experiments on animals act entered into force on 5 February 1997 http://www.nca-nl.org/ |
| Norway Ministry of Agriculture | Norwegian Animal Welfare Act, 1974 New version of June 16, 1995 http://www.dyrebeskyttelsen.no/english/law.shtml |
| Republic of Czechia Czech National Assembly | “Law for the Protection of Animals” no 246 Sb, 15. April 1992, inclusive the amendments no. 162 Sb, 19. May 1993 http://www.nca-nl.org/ |
| South Korea 1. Ministry of Agricul- ture, Fishery and Forestry 2. Ministry of Health and Welfare | 1. “Animal Protection Act”, May 1991 2. “Pharmaceutical Affairs Law” Korean Good Laboratory Practice Guidelines for Breeding and Caretaking of Test Animals |
| Spain | Real Decreto 223/1988 http://www.boe.es |
| Sweden The Swedish National Board for Laboratory Animals (CFN) Ministry of Agriculture, Stockholm | National Board for Laboratory Animals Ordinance Amending the Board’s Ordinance (LSFS 1988:45, as last amended by SFS 1998:1344 of October 22, 1998) Containing Regulations and General Recommendations Concerning Ethical Examinations of the Use of Laboratory Animals for Scientific Purposes, etc. http://www.nca-nl.org/ |
| Switzerland | “Tierschutzgesetz vom 9. März 1978 (TSchG), under revision in 2006 “Tierschutzverordnung”, 27. Mai 1981 http://www.bvet.admin.ch/ |
| Taiwan Council of Agriculture; In future: Provincial/ Municipal Governments | Taiwan Animal Protection Law, November 4, 1998. Very generally, so that additional regulations are going to be required. http://www.gio.gov.tw/info/98html/aplaw.htm |

Table 1 (continued)

| Country, Institutions | Legislation |
|----------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| United Kingdom | “Protection of Animals Act”, 1991 “Animals (Scientific Procedures) Act”, 1986 http://scienceandresearch.homeoffice.gov.uk/animal-research/legislation/ |
| U.S.A. U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Animal Care (AC) | Guide for the Care and Use of Laboratory Animals, January 1996 http://www.nap.edu/readingroom/books/labrats/ The Animal Welfare Act, signed into law in 1966, amended four times (1970, 1976, 1985, 1990), can be found in United States Code, Title 7, Sections 2131 to 2156 http://www.nal.usda.gov/awic/legislat/usdaleg1.htm |
| Most countries | Local ethical commissions at universities and other institutions of biomedical research Useful Internet-links on international animal protection: http://www.nca-nl.org/ http://www.animallaw.info/ http://www.nal.usda.gov/awic/legislat/internat.htm http://www.uni-giessen.de/tierschutz/ |

from those of single or repeated blood collections from a conscious animal. Terminal blood collection under anesthesia allows the use of techniques which are not acceptable for non-terminal blood collections.

Q.2.2 Aspects of Animal Welfare

Minimizing any pain and distress in laboratory animals during the procedure have to be as important as achieving the desired experimental results. This is important not only for humanitarian reasons but also as part of good scientific practice. Blood collection may be stressful to the animal due to the handling and the discomfort associated with a particular technique. Many biochemical and physiological changes are associated with stress which affect the results, e. g. increases in the blood levels of catecholamines, prolactin and glucocorticosteroids can influence certain metabolic parameters, such as glucose, as well as the counts of erythrocytes, white cells, and packed cell volume. Therefore, stress should be reduced to an absolute minimum if it is not possible to avoid it at all, this is not only in the interest of animal welfare but also in the interest of good science to obtain representative data. To minimize stress during blood collection, e. g. from dogs or cats, it may be helpful for the animal as well as for the operator first to do some dummy runs and provide rewards to the animal.

During non-terminal blood collection it is important not to withdraw too much blood which could reduce total blood volume and lead to false results. A reduced total blood volume is accompanied by a reduced hemoglobin content and oxygen transport capacity (Gainer et al. 1955) as well as by a fall in blood pres-

sure, and an increase in the concentrations of stress-related hormones. It may be further accompanied by other factors such as necrosis of the gastric mucosa.

The welfare of the individual animal should not be endangered by removal of too large a volume of blood or by too frequent collections. This may be the case more often when small laboratory animals, e. g. mice, gerbils, rats or hamsters are used. In these cases the study protocol should be adapted to use more animals to minimize distress for the individual animal.

Q.2.3 Total Blood Volume

The total blood volume is very difficult to determine (McGuill et al. 1989) and depends on species, sex, age and health as well as nutritional condition. Total blood volume is smaller in larger animals than in smaller animals of the same species in relation to body weight. It is also smaller in older and obese animals compared to normal weight and young animals. Total circulating blood volume is in the range of 55–70 ml/kg body weight.

Q.2.4 Terminal Blood Collection

Terminal blood collection represents (i) exsanguination as a single process of blood removal to collect as much blood as possible and (ii) multiple blood sampling during a terminal experiment under general anesthesia. Basically, exsanguination should only be performed after the animal has been rendered unconscious by another method, e. g. physical stunning or general anesthesia. This is due to the fact that stress occurs

with extreme hypovolemia and accessing deeper blood vessels causes pain. Due to the anesthetized condition of the animal and the terminal nature of the experiment, methods can be used for exsanguination which can never be recommended for non-terminal blood collections with recovery of the animal. These include

- blood withdrawal from the V. cava caudalis or the aorta after laparotomy when as much blood as possible should be removed in a sterile manner,
- exsanguination after decapitation, incision of the jugular vein or carotid artery or techniques in the slaughterhouse, when a non-sterile collection is possible,
- retro-orbital bleeding of smaller laboratory animals like mice, gerbils, hamsters and rats which can also be a method of exsanguination.

Q.2.5

Non-terminal Blood Collection

Non-terminal blood collections can be differentiated into single and multiple blood withdrawals. Possible peripheral veins for blood withdrawal are listed in Table Q.2.

Q.2.5.1

Single Blood Removal

A single withdrawal of up to 15% of total blood volume does not influence the well-being of the animal.

However, the removal of 15 to 20% might be accompanied by side effects such as fall in cardiac output or blood pressure. Haemorrhagic shock can be induced by the withdrawal of 30–40% of total blood volume and the loss of 40% causes mortality in up to 50% of pigs and rats (McGuill et al. 1989).

A single removal of up to 15% of total blood volume may be repeated after 3–4 weeks from normal and healthy animals with no detectable adverse effects. This does not mean that the animal does not experience any adverse effects, but it does not show any.

Symptoms of hypovolaemic shock are fast pulse, pale mucous membranes, hyperventilation and a sub-normal body temperature including cold skin and extremities. In these animals therapeutic intervention consists of volume substitution with warm isotonic intravenous infusion.

Q.2.5.2

Multiple Blood Removal

Multiple withdrawal of blood samples should not exceed 1% of total blood volume every 24 h (0.6 ml/kg/d). More frequent withdrawals and/or removal of larger volumes of blood causes anemia.

Symptoms of anemia are pale mucous membranes of the conjunctiva or inside the mouth, intolerance to exercise and an increased respiratory rate in cases of severe anemia. Anemia can be easily detected by de-

Table 2 Blood vessels for venous blood withdrawal

| Species | V. coccygica + tt | V. auricularis | Orbital venous | V. jugularis | V. cephalica V. saphena | V. femoralis | V. mammarica |
|---------------|-------------------|----------------|----------------|--------------|----------------------------|--------------|--------------|
| Mouse | cc + cc | – | a | – | – | – | – |
| Gerbil | cc + cc | – | aa | a | – | – | – |
| Hamster | – | — | aa | a | – | – | – |
| Rat | ccc + cc | – | a | aa | – | – | – |
| Guinea pig | – | c | – | a | – | – | – |
| Rabbit | – | ccc | – | c | – | – | – |
| Cat | – | – | – | aa/c | ccc | cc | – |
| Dog | – | – | – | ccc | ccc | a | – |
| Rhesus monkey | – | – | – | aa/c | ccc | cc | – |
| Pig | – | iii | – | iii/cc(cvc) | – | – | – |
| Sheep | – | – | – | ccc | c | – c | – |
| Goat | – | – | – | ccc | c | – | – c |
| Cattle | cc | c | – | ccc | c | – | – cc |
| Horse | – | – | – | ccc | – | – | – |

c/cc/ccc conscious animal, c/i/a possible alternative, i/ii/iii immobilized animal, – not recommended or impossible, a/aa/aaa anaesthetized animal, cvc cranial vena cava, ccc/iii/aaa recommended route, tt amputation tail tip, cc/ii/aa acceptable route repetition of letters indicates the preferred condition

termination of erythrocyte cell count and packed cell volume (haematocrit), hemoglobin level as well as reticulocyte count in a blood sample. In case of anemia the animal should be treated with iron and vitamin B12 and should be monitored for the above mentioned blood parameters during therapy until normal values are reached again.

Q.2.6

Technical Aspects of Blood Removal

A common method in mice and rats for collecting up to 0.1 ml capillary blood is to remove the tip of the tail. For repeated blood sampling the blood clot on the tail has to be removed to get fresh capillary blood. This method is sufficient for multiple blood collections to determine, e. g. blood glucose or total radioactivity after the administration of radiolabeled drugs. In tailless animals such as guinea pigs and hamsters, cardiac puncture under general anesthesia may be the preferred technique.

Blood collections from larger animals will preferably be performed from a superficial vein. The person holding the animal and raising the vein plays a key role in collecting blood without undue stress to the animal by talking to and stroking the animal. Some animals, e. g. dogs and some primates, may be trained to present a limb for blood removal without the use of any physical restraint.

It is important to locate the vessel accurately before insertion of the needle or the catheter. In most cases obstruction of the venous return is necessary for distension of the vessel and to successfully insert the needle. The bore of the needle should be as large as possible to ensure rapid blood withdrawal with minimal risk of blood clotting within the needle. When the sample is taken too quickly by a syringe, the vein will collapse. After the needle has been withdrawn, continuous pressure should be applied immediately to the puncture site and maintained for at least 30 s. The animal should be monitored 15 min later to check for after-bleeding.

Q.2.6.1

Permanent Venous Cannulation

For multiple blood collections a permanent venous access by chronic cannulation is often recommended. In most cases, particularly in rats, it is necessary to restrain the animal in harnesses or jackets to prevent it from damaging or removing the cannula. In these cases the signs of stress are often apparent by an increase in

serum levels of stress hormones. However, a few days after implantation of catheters, hormone levels are normal in restrained rats (Tsukamoto et al. 1984; Wiersma and Kastelijn 1985). Such animals are usually housed alone and the tethering restricts normal movements such as lying on the back and rolling over. Such restrictions may be considered as potential sources of stress. This can be prevented by having the catheter exit the back of the animal for only 2 cm and capping it with a steel needle. At the time of the experiment, a longer catheter is attached for blood collections.

A simple device for serial blood collection has been described by Sir-Petermann et al. (1995).

One has to balance very carefully the distress and discomfort of the individual animal with a permanent cannulation under restraint conditions for a longer period with multiple blood samplings without permanent cannulation. In the first case, multiple values from the same animal can be obtained showing perhaps individual differences among a group of animals. In the latter case it may be necessary to use a larger number of animals but there is less discomfort for the individual animal.

Short-term cannulation (less than a day) of a peripheral blood vessel in larger animals is easy to perform. A butterfly needle can be inserted under aseptic conditions and multiple blood samples can readily be collected. Long-term cannulation (longer than two days) in larger and smaller animals often presents complications such as blockage of the cannula by thrombi. The infusion and administration of substances via the permanent cannula are much easier than the removal of blood. Thrombi attached to the end of the cannula function as a one-way valve. Clotting can be prevented by repeatedly filling the catheter with saline containing heparin.

Q.2.6.2

Retro-Orbital Bleeding

Blood sampling by orbital puncture is a controversial technique. The puncture of the orbital venous plexus is often performed in tailless animals, e. g. hamsters. This technique is also used in rats and mice, when larger volumes are required which cannot be obtained from the tail vein. Basically, retro-orbital bleeding should always be performed under anesthesia. Pasteur pipettes, micropipettes or microcapillary tubes are used and pushed with a rotating movement through the conjunctiva laterally, dorsally or medially of the eye to the back wall of the orbit. In general, inflammatory reactions can be seen histologically in the puncture

track four days after puncture. After 4 weeks the lesions have healed without detectable scars (van Herck et al. 1992). However severe side-effects such as retro-orbital haematoma with subsequent pressure on the eye cannot be completely excluded. This pressure can damage the optical nerve. The animal may be unable to close its eye. Bleeding from the orbital venous plexus should only be performed with recovery of the animal in exceptional circumstances when there is no other method available. The technique should be performed only by a well-trained staff and only one eye should be used.

Q.2.6.3

Cardiac Puncture

The collection of blood by cardiac puncture has been performed in guinea pigs, gerbils and hamsters. In these species it is difficult to collect blood by alternative methods except retro-orbital bleeding. In general, cardiac puncture should be performed under general anesthesia with atropine as premedication to prevent cardiac arrhythmia. If cardiac puncture is used for a non-terminal blood withdrawal with recovery, the animal has to be separated from other animals until it is fully conscious. It should be carefully watched for adverse effects and sacrificed if found in distress due to complications like bleeding into the pericardium or into the thorax.

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Q.3

Anesthesia of Experimental Animals²

Q.3.1

Introduction

In biomedical research, experiments should only be done with a conscious animal if it is not possible to do the study in an anesthetized one. Anesthetic conditions should always be chosen to exclude stress, discomfort and pain for the animal which could have negative influences on the pharmacological results and reproducibility of the data. Therefore, an experimental design causing minimal discomfort to the animal is always preferable. This is important not only for humanitarian reasons but also for good scientific practice.

Many pharmacological experiments are performed under anesthesia:

- terminal experiments under anesthesia followed by euthanasia,
- experiments under anesthesia with recovery at the end of the study, and
- experiments in which an animal is surgically prepared under anesthesia and continuation of the experiment occurs with the conscious animal after recovery.

Generally, two possibilities exist for immobilization of aggressive animals and to prevent escape: (i) physical restraint (e. g. immobilization cages or immobilization tubes) and (ii) chemical restraint with anesthetic compounds. As a rule of thumb it is recommended to use physical restraint for animal studies in which no anesthesia would be used in comparable studies in man. Physical restraint can be used for short and painless interventions like administration of substances or blood sampling from a vein.

In general, physical restraint produces fear, distress and anxiety in experimental animals with the result of stress symptoms which could affect the results of the study. To minimize pain for the animals, to obtain correct and reproducible results and to protect the handlers from aggression by the animals, it is often necessary to use chemical restraint. However, the chemical used can affect the biochemistry or physiology of the animal.

It is possible to anesthetize special areas of the animal (local anesthesia) or the whole animal (general anesthesia). Local anesthesia plays only a minor role

²By J. Maas, A.W. Herling, K. Seeger (first edition), revised by U. Albus (2nd and 3rd edition).

for experimental animals as compared to general anesthesia.

Q.3.2

Local Anesthesia

Local anesthesia is the regional and reversible elimination of pain with chemical compounds. Circulatory, pulmonary and renal functions are not disturbed and the animals are conscious. Surface anesthesia has to be distinguished from the anesthesia produced after a local injection. The most common compounds for surface anesthesia are tetracaine and proparacaine. Procaine, butanilicaine, lidocaine, mepivacaine and etidocaine are commonly used injectable local anesthetics. Local anesthesia is only recommended for gentle and calm animals (cattle, sheep). For most laboratory animals, general anesthesia is the method of choice.

Q.3.3

General Anesthesia

Q.3.3.1

Preparation

It is very important to check the general condition of the animal prior to anesthesia. This check should include a clinical examination (inspection, auscultation, palpation) of the animal concerned. Sometimes it could be useful to perform a few laboratory tests, e. g. hematocrit, hemoglobin, pH-value of blood and acid/base parameters.

Those animals with a vomiting reflex should be fasted prior to anesthesia. Most animal species should be fasted for a period of at least 12 h but pigs and cattle for at least 24 h. Water can be offered during the fasting period ad libitum.

Q.3.3.2

Premedication

Premedication is recommended prior to anesthesia for easier administration of the anesthetic and for elimination of side effects of the anesthetic used, such as disturbing autonomic reflexes.

Q.3.3.2.1

Hydration and Base Excess

Based on hematocrit, hemoglobin and erythrocyte values, the hydration of the animal should be normalized prior to anesthesia. Infusions of glucose or Ringer solution can be used for this purpose. To check the success of the treatment repeated determinations of the above mentioned values are necessary.

In cases of acidosis (pH of blood < 7.36), treatment of the animals with NaHCO₃ is recommended. If measurement of base excess is possible, the amount of NaHCO₃ can be determined from the following formula:

$$\begin{aligned} \text{Dose NaHCO}_3 \text{ (ml)} \\ = \text{g body weight} \times \text{base excess}/0.6 \end{aligned}$$

Q.3.3.2.2

Atropine

To avoid cardiopulmonary problems and to decrease saliva production, atropine should be administered intramuscularly prior to general anesthesia. The recommended dose varies considerably and is usually between 0.05 and 0.1 mg/kg body weight. Cats and rodents have a higher activity of atropine-esterase in the liver, and these species need higher amounts of atropine (up to 0.25 mg/kg).

Q.3.3.2.3

Sedation and Pain Elimination

Indications for sedation and elimination of pain are to calm the animals and to stabilize the autonomic nervous system.

For sedation the following compounds are used:

- minor tranquilizers without autonomic effects:
- meprobamate, diazepam,
- major tranquilizers with autonomic side effects:
- propionyl-promazine, acetylpromazine, azaperone, dehydrobenzperidol, xylazine, detamidone.

Anaesthesia does not necessarily lead to analgesia (elimination of pain). Although general anaesthesia produces loss of consciousness and pain is not perceived, the noxious stimuli will be transmitted to the CNS and will develop central hypersensitivity, leading to a postoperative heightened perception. To reduce the degree of central hypersensitivity analgesics have to be administered before noxious stimulation begins (preemptive analgesia). Analgesia should also reduce or eliminate peripheral inflammation, which aggravates central hypersensitivity.

For analgesia, the opioids used are mainly:

- methadone, meperidine, fentanyl.

In most species (dog, rabbit, guinea pig) a sedative effect is to be observed after administration of these compounds. In other species (pig, cat) an excitatory effect can occur.

Minor tranquilizers, major tranquilizers and analgesic compounds are often used in common with anes-

Table 3 Anesthesia of experimental animals (values are in mg/kg)

| Species | Premedication | Sedation | Short anaesthesia | Medium anaesthesia | Long anaesthesia |
|---------------------|--------------------------|------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|
| Rat | Atropine (0.2 s.c.) | Diazepam (2.5 i.m.) | Alfentanyle + Etomidate (0.03 + 2 i.m.) or Inhalation (Isoflurane) | Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone (50 i.p.) | Xylazine + Ketamine (16 + 100 i.m.) or Urethane (1500 i.m.) |
| Mouse | Atropine (0.1–0.25 s.c.) | Diazepam (5 i.p.) | Alfentanyle + Etomidate (0.03 + 2 i.m.) or Inhalation (Isoflurane) | Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone (50 i.p.) | Xylazine + Ketamine (16 + 100 i.m.) |
| Hamster | Atropine (0.1–0.2 s.c.) | Diazepam (5 i.p.) | Inhalation (Isoflurane) | Xylazine + Ketamine (5 + 50 i.m.) or Pentobarbitone (35 i.p.) | Xylazine + Ketamine (10 + 200 i.m.) |
| Guinea pig | Atropine (0.1–0.2 s.c.) | Diazepam (2.5–5 i.m.) | Inhalation (Isoflurane) | Xylazine + Ketamine (2 + 80 i.m.) | Xylazine + Ketamine (4 + 100 i.m.) or Pentobarbitone + Chloralhydrate (30 i.p. + 300 i.v.) |
| Rabbit | Atropine (0.1–0.2 s.c.) | Diazepam (1–5 i.m.) | Inhalation (Isoflurane) | Xylazine + Ketamine (5 + 25–80 i.m.) | Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone + Chloralhydrate (30 i.p. + 300 i.v.) |
| Cat | Atropine (0.05–0.2 s.c.) | Diazepam (0.2–1 i.m.) | Acetylpromazine (0.5–1 i.v./i.m.) or Propionylpromazine (0.5–1 i.v.) or Xylazine (2 i.m.) | Xylazine + Ketamine (2 + 10 i.m.) or Ketamine (5 i.v.) or Inhalation (Isoflurane) | Pentobarbitone (35 i.v./i.p.) |
| Dog | Atropine (0.05 s.c.) | Xylazine (3 i.m.) or Acetylpromazine (0.5 i.m.) or Propionylpromazine (0.5 i.m.) or Droperidol (1 i.m.) or Diazepam (1 i.m.) | Thiopental (17 i.v.) or Metomidate + Fentanyl (4 + 0.005 i.m.) or Alfentanil + Etomidate (0.03 + 1 i.m.) or Inhalation/Intubation (Isoflurane) | Xylazine + Methadone (2 + 1 i.m.) or Xylazine + Ketamine (2 + 10 i.m.) or comb. with Diazepam (0.6 i.m.) or Propionylpromazine + Methadone (0.5 + 1 i.v.) or Acetylpromazine + Methadone (0.5 + 0.5–1 i.v.) | Pentobarbitone (30 i.v.) or Xylazine + Ketamine (2 + 15 i.m.) or Intubation (Isoflurane) |
| Pig | | Azaperone (1–2 i.m.) or Chlorpromazine (1–2 i.m.) | Thiopental (10 i.v.; 5% solution) | Azaperone + Metomidate (0.05–5 + 2.5–5 i.m. + i.v./i.p.) or Tiletamine + Zolazepam + Xylazine (2 + 2 + 0.5–1 i.m.) | Pentobarbitone (10–25 i.v.) |
| Sheep Goat | | Xylazine (0.05–0.1 i.m.) or Diazepam (2 i.m.) | Xylazine + Ketamine (1–2 + 5–10 i.m.) or Thiopental (7.5–10 i.v.) | Pentobarbitone (20–30 i.m.) | Ketamine after pretreatment with Xylazine/ketamine 3–4 h or Intubation (Isoflurane) |
| Monkey ^a | Atropine (0.05–0.1 s.c.) | Diazepam (1.0 i.m.) or Xylazine (1–2 i.m.) or Ketamine (10–30 i.m.) | Inhalation/Intubation (Isoflurane) | Xylazine + Ketamine (2 + 10 i.m.) | Pentobarbitone (20–30 i.v./i.p.) or Intubation (Isoflurane) |

^a Anaesthesia of monkeys depends very much on the monkey species.

thetics. A compilation of such combinations is summarized in Table Q.3.

Q.3.3.3

Course of Anesthesia

The animal has always to be observed very carefully during anesthesia. Various systems can be checked with technical equipment, e.g. circulatory system (heart rate, pulse, blood pressure, ECG, peripheral perfusion, temperature) or pulmonary system (respiratory rate).

A very important procedure during anesthesia is the determination of the depth of anesthesia. There are four stages of anesthesia:

- I. Stage of analgesia (from the first effect to unconsciousness):
 - heart and respiratory rate increase, normal dilation of pupils.
- II. Stage of excitation (from the beginning of unconsciousness to the start of regular respiration): respiration irregular, dilated pupils, increased motor reflexes, nystagmus, opisthotonus.
- III. Stage of tolerance (from the beginning of regular respiration to the termination of spontaneous respiration):

This stage is divided into four steps:

 - A) regular respiration, narrow pupils, most reflexes present
 - B) skeletal muscles relaxed, narrow pupils, no eyelid reflex, corneal reflex present, flat respiration, good analgesia.

This is the optimal stage of anesthesia for surgery
 - C) only corneal reflex present, respiration very flat, pupils dilated
 - D) no reflexes, respiration very flat, pupils very dilated
- IV. Stage of asphyxia (after termination of the spontaneous diaphragmatic respiration):
- V. no reflexes, no respiration: danger of death, immediate use of antidotes is necessary to prevent death.

By using combinations of different anesthetics – mainly by using combinations with muscle relaxing agents – the reactions of animals will differ from this scheme.

Q.3.3.4

Routes of General Anesthesia

In general, there are two different routes to induce general anesthesia: (i) injection and (ii) inhalation anesthesia. Sometimes combinations of both routes are used.

The decision for one or the other route depends on the animal species, the purpose of the study and the necessity of control during anesthesia.

Q.3.3.4.1

Injection

By using this route of anesthesia the narcotic compound is dissolved in a liquid. The route of administration can be intravenous, intramuscular, subcutaneous or intraperitoneal. The mostly frequently used compounds are mentioned below:

Barbiturates

There are three groups of barbiturates: long acting, short acting and very short acting barbiturates. For laboratory animals short and very short acting barbiturates are used predominantly (sodium pentobarbitone, thiopental, hexobarbital).

Barbiturates are metabolized in the liver and mainly excreted via the bile. They are very fat soluble. Their short duration of action is caused by a distribution into adipose tissue. Fat represents a large compartment for these compounds with a relatively slow excretion. This can lead to prolongation of the narcotic effects after repeated dosing. The dosing of barbiturates should be adjusted according to the observed reactions of the individual animal as there are individual differences due to age, body weight, size, fat content and general condition of the animal. Barbiturates are not analgesic and should not be given without opioids.

Chloralhydrate

Chloralhydrate is a relatively old soporific compound. By using it for anesthesia cardiovascular side effects are often observed. The range of dosing is very narrow. Its use for laboratory animals is therefore limited. Intraperitoneal injections in rats can lead to paralysis of the ileus.

Combinations of Analgesic with Neuroleptic Compounds

This method is often used for dogs and rodents. Strong analgesics (morphine, methadone, meperidine, fentanyl) are combined with neuroleptics like phenothiazine, acetylpromazine or butyrophenone. The anaesthesia can be rapidly terminated by available antagonists.

Ketamine

Ketamine is a neuroleptic compound with a very fast onset of action following intramuscular administration. It can be used for nearly all species. A side effect of this compound is an increased tonus of skeletal

muscles but this can be prevented by the simultaneous administration of xylazine or diazepam.

Hypnotic Agents

Hypnotics are compounds which produce a very deep sleep without analgesia (metomidate). Therefore combination with neuroleptic compounds is recommended (e. g. combination azaperone with metomidate for pigs). As a single compound, metomidate can only cause anesthesia in birds.

Xylazine

Xylazine is frequently used for anesthesia in combination with other substances (Table Q.3). As a single compound it is only used to produce anesthesia in cattle.

Urethane

Urethane was formerly used as a hypnotic agent. It can, at the appropriate dose, produce a long acting (about 10 h) anesthesia in rats. Urethane is liver toxic and therefore its use is limited to some pharmacological models in which liver metabolism is of no importance. Due to its carcinogenic properties it should not be used anymore.

The important criteria of anesthesia are sedation, unconsciousness, analgesia and relaxation. These cannot be achieved with a single compound. Therefore, a combination of different compounds is necessary. The most common combinations for different species are listed in Table Q.3 with respect to the duration of anesthesia: short (up to 30 min), medium (up to 120 min) and long anesthesia (longer than 120 min).

Q.3.3.4.2

Inhalation

Inhalation anesthesia is more common for the bigger laboratory animals such as dogs, cats, sheep, goats and monkeys. However, it has gained an increased importance in small laboratory animals like rodents. The advantages of this form of anesthesia are the possibilities of controlling exactly the depth of anesthesia and of fast management of complications.

The parts of an inhalation system include:

- Bottle with oxygen (blue bottles)
- Valve to regulate pressure (reduces the pressure of the oxygen-bottle)
- Flowmeter (monitors the gas flow to the animal)
- Evaporator (evaporation of liquid anesthetic compounds)

- Oxygen-bypass (fast supply of oxygen to the animal in case of need)
- Tube to the system

Different techniques are used for laboratory animals:

Technique of Insufflation

Administration of anesthetic compounds is performed via a mask. Expiration occurs into the air of the room. Advantages are the simple procedure without valves and CO₂-absorber, and the very small dead volume of the system. Disadvantages are the waste of compounds, drying of the trachea of the animals, the impossibility of checking the respiration volume and the expiration of narcotic compounds into the room air (jeopardy to the staff).

Open System

Inspired and expired gases are separated by a valve. The inspired air consists of the fresh mixture of gases. The expiration reaches completely the atmosphere. The “Stephen slater” is the most used system of this group. It is recommended for smaller animals.

Half-Closed and Closed Systems

In closed systems all of the expired air passes to a CO₂ absorber. The CO₂ is removed chemically and the air is inspired again with newly evaporated anesthetic compounds mixed with oxygen. In a half-closed system, part of the expired air reaches the atmosphere. Advantages of closed systems include the economic benefit, the decrease of fluid and body heat loss from the animal and no risk to the laboratory staff. Disadvantages are the necessity to change the absorber every 8–10 h during anesthesia, the production of heat and the increase of resistance to breathing.

Summary

If it is possible inhalation should be done by intubating the animal. The risk of aspiration of stomach contents with the danger of an aspiration pneumonia can then be minimized. It is very important to use a tube with the correct diameter and length. An animal should be unconscious for intubation (see Table Q.3). In order to avoid gulp or cough reflexes it is recommended to administer succinylcholine, a muscle relaxant. Atropine can also be administered to decrease saliva production. Generally, all methods of injection anesthesia mentioned (Table Q.3) can be combined with an inhalation method. Such a “balanced anesthesia” is recommended for long and highly sophisticated operations.

| Compound | Conc. with N ₂ O/O ₂ (%) | Conc. without N ₂ O/O ₂ (%) | MAC |
|----------------|------------------------------------------------|---------------------------------------------------|------|
| Methoxyflurane | 0.4–1 | 3.5 | 0.23 |
| Isoflurane | 1.0–2.5 | 1.5–3.5 | 1.4 |
| Enflurane | 0.5–1.5 | 2 | 2.2 |

Table 4 Inhalation compounds and their characteristics

MAC: minimal alveolar concentration for an anesthetic effect.

Q.3.3.4.3 Inhalation Compounds

The inhalation mixture has to include 21% oxygen. Sometimes it is better to administer 33% oxygen. Isoflurane, methoxyflurane and enflurane are widely used compounds for inhalation anesthesia. Ether is not longer recommended for anesthesia, due to the hazard of explosion and fire and because it is highly irritating to the respiratory tract. By using a mixture of N₂O and O₂ the amount of the evaporated compounds can be reduced drastically (Table Q.4).

Q.3.3.5 Termination of Anesthesia

Inhalation anesthesia can be stopped by removing the supply of evaporated compounds. To hasten the elimination of anesthetic compounds, the concentration of oxygen in the system can be increased for a period of five min.

The elimination of injected compounds is difficult to influence. It may be possible to accelerate metabolism of the anesthetic by using agents which stimulate metabolism in the liver and excretion by the kidney.

It is very important to check the body temperature of the animal during and after anesthesia. In cases of low body temperature the use of heating lamps or pads is necessary. After termination of anesthesia the animals go through the same phases as mentioned above but in the reverse order (tolerance, excitation, analgesia).

During anesthesia it might be necessary to stimulate respiration or circulation. Stimulatory agents for respiration are doxapram, pentamethylentetrazole, nikethamide, methetarimide, lobeline or micoren. Stimulatory agents for circulation are adrenaline, efortil, dopamine and ephedrine. The application of pure oxygen via a mask is also recommended during an injection anesthesia. Antidotes to morphine and its derivatives are morphine-antagonists like naloxone. Yohimbine is an antagonist of xylazine. The antidote for diazepam is flumazenil. There are no direct antagonists for ketamine and barbiturates.

Q.3.4 Postoperative Analgesia

To effectively reduce pain in animals a pain assessment has to be made using behavior, stress response etc. Pain assessment will be facilitated by

- a good knowledge of the species specific behaviors of the animal being assessed
- a knowledge and comparison of the individual animal's behaviour before and after the onset of pain (eg pre- and post-operatively)
- the use of palpation or manipulation of the affected area and assessment of the responses obtained
- examination of the level of function of the affected area: e. g. leg use following injury or limb surgery, together with a knowledge of any mechanical interference with function
- the use of analgesic regimens or dose rates that have been shown to be effective in controlled clinical studies, and evaluation of the change in behaviour this brings about
- a knowledge of the non-specific effects of any analgesic, anaesthetic or other drugs that have been administered

Analgesics can be broadly divided into two groups, the opioids or narcotic analgesics and the non-steroidal anti-inflammatory drugs (NSAID). Clinical pain involves several pathways, mechanisms and transmitter systems. To provide the most effective pain relief, drugs of different classes should be applied, acting on different parts of the pain system, for example combining opioids and NSAIDs.

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Q.4 Euthanasia of Experimental Animals³

Q.4.1

Introduction

Biomedical research needs animals. This is most obvious in case of *in vivo* animal experiments. However, for other scientific purposes, e. g. *in vitro* studies, biological material is also necessary to study enzymes, membranes, receptors, cells, tissues, or organs which are obtained from dead animals. Therefore, animals have to be sacrificed in biomedical laboratories (i) at the end of an *in vivo* experiment, (ii) during experiments where sacrifice of the animals is not part of the study but must be done when pain, distress and suffering exceed acceptable levels or if it is likely for the animal to remain in pain or distress after cessation of the experiment, and (iii) to provide biological material for *in vitro* studies.

The following remarks are a summary of the *Recommendations for Euthanasia of Experimental Animals* of the *Commission of the European Communities* (1993) and the *Guidelines for Skillful and Human Euthanasia of Laboratory Animals* (1993) of Switzerland.

Q.4.2

Euthanasia

Euthanasia means a gentle death and should be regarded as an act of a human method of sacrificing an animal with a minimum of physical and mental suffering. The method of euthanasia should be appropriate for the species and the age of the animals. The method should be painless, avoid excitement and achieve rapid unconsciousness and death. Additionally, the method should be reliable, reproducible and irreversible.

Prior to euthanasia, it is important to recognize symptoms of fear, distress and anxiety; these symptoms are species specific. Depending on the species these symptoms may include distress vocalisation, attempts to escape, aggression, freezing, salivation, urination and defecation. Distress vocalization and release of certain odors or pheromones by a frightened animal may cause anxiety in other animals housed nearby. In this context it has to be stressed that many vocalisations of animals are in a range of frequencies

which are out of the human hearing range. Therefore, animals should not be present during euthanasia of other animals, especially of their own species. If possible, an animal should not be killed in a room where other animals are housed, in particular in case of a bloody method of euthanasia, e. g. decapitation.

Euthanasia usually requires some physical control over the animals. Suitable control minimizes pain, distress, fear and anxiety in the animal and depends on animal species, size, state of domestication and method of euthanasia. Gentle handling, stroking and talking to the animal during euthanasia often have a calming effect on many animals. The use of sedating and immobilising drugs may be necessary in those cases, where capture or restraint may cause pain, injury or anxiety to the animal.

The person performing euthanasia is the most relevant factor during sacrificing an animal in order to cause a minimum of pain, fear and distress. A suitable method of euthanasia can be extremely harmful to the animal if it is badly performed. All persons performing euthanasia should be well trained, demonstrate professionalism and be sensitive to the value of animal life.

After euthanasia it is essential to confirm death. Signs of death are cessation of heartbeat and respiration, and absence of reflexes. Death must be guaranteed by exsanguination or removal of the heart, destruction of the brain, decapitation, evisceration or the presence of rigor mortis.

Methods for euthanasia of laboratory animals can be separated into physical and chemical methods.

Q.4.2.1

Physical Methods Recommended for Euthanasia of Laboratory Animals

Physical methods are stunning (concussion, electrical stunning, and stunning with a captive bolt), cervical dislocation, decapitation, and microwave irradiation. The different methods of stunning as well as cervical dislocation cause a rapid loss of consciousness which must be followed immediately by a method to force and guarantee death of the animal.

Concussion may be sufficient in smaller animals, e. g. rodents, to achieve unconsciousness and is performed by a blow to the head. Electrical stunning is a common method in the slaughterhouse predominantly for pigs. Only specific equipment must be used for this method either in the slaughterhouse or in laboratory. Stunning with a captive bolt is also a common and an effective method for larger animals in the slaughterhouse to achieve unconsciousness. Adapted equipment can also be used for larger rabbits in biomedical labo-

³By A.W. Herling (1st edition), revised by U. Albus (2nd and 3rd edition).

ratories. The correct localisation of the captive bolt is important in order to achieve immediate destruction of the brain. Cervical dislocation destroys the brainstem but the large vessels to the brain are often intact. All these methods have to be followed immediately by an act to force and guarantee death, e. g. exsanguination, removal of the heart or destruction of the brain.

During the decapitation process the head is separated from the neck which causes an immediate interruption of the blood circulation to the brain and a fall in blood pressure in the brain with subsequent loss of consciousness. This is valid only for warm-blooded animals. In cold-blooded vertebrates it is recommended to stun the animals prior to decapitation due to their higher resistance against anoxia. For decapitation of smaller laboratory animals specific guillotines have been developed.

Euthanasia by microwave irradiation is used by neurobiologists for fixation of brain metabolites without destruction of brain anatomy. Only specific equipment developed for this purpose must be used (no domestic microwave ovens). It is essential to localize correctly the microwave beam onto the brain of the animal.

Q.4.2.2

Chemical Agents Recommended for Euthanasia of Laboratory Animals

Many chemicals can cause death due to their toxicity, but only a few are recommended for euthanasia. The most suitable chemicals for euthanasia are certain anesthetics in overdose. In this case, the anaesthetic agent causes unconsciousness, followed by death.

Volatile anesthetics such as halothane, enflurane, isoflurane and methoxyflurane should only be used in a gas scavenging apparatus. Carbon dioxide at high concentrations of 80 to 100% causes unconsciousness within a few seconds.

Injectable anesthetics, predominantly barbiturates such as sodium pentobarbitone, are the most widely used and the most appropriate agents for euthanasia for most animals. Three times the anesthetic dose causes generally rapid unconsciousness and death. Intravenous injection is the most reliable and rapid route. Intraperitoneal injection may also be used in smaller rodents but it needs more time for death to occur. The intracardial and intrapulmonary administration can only be recommended in unconscious animals, because it is painful and, in the case of intracardial injection, difficult to perform successfully on the first attempt.

The agent T61 is a mixture of a local anesthetic, a hypnotic and a curariform component. It is used

only intravenously. Due to the curareform component it is not allowed in some countries but it has been demonstrated that unconsciousness and neuromuscular blockade occur simultaneously in dogs and rabbits. Nevertheless, prior sedation should be performed if possible.

Q.4.2.3

Methods and Agents Not to Be Used for Euthanasia of Laboratory Animals

Physical methods not to be used for euthanasia are exsanguination, rapid freezing, pithing, decompression, hyperthermia, hypothermia, asphyxia, drowning and strangulation. Chemicals not to be used are carbon monoxide, nitrogen, nitrous oxide, cyclopropane, chloroform, trichloethylene, hydrogen cyanide, magnesium sulfate, potassium chloride, nicotine, strychnine, chloral hydrate, and ethanol. Some of the above mentioned chemicals are not recommended for euthanasia because they are extremely noxious and dangerous to the experimenter.

Neuromuscular blocking agents such as curare, succinylcholine or suxamethonium which do not cause rapid unconsciousness prior to death should also not be used. Ketamine is a very good anesthetic with a wide therapeutic safety margin for most animal species. Therefore, it is unsuitable for euthanasia.

However, non-acceptable methods of euthanasia can be used if animals are anaesthetized or rendered insensible and unconscious by a recommended method. This is used for, e. g. exsanguination, rapid freezing, and pithing. Exsanguination must not be performed in sight or smell of other animals. Rapid freezing is important to minimize enzymatic processes prior to subsequent biochemical determinations in tissues and organs. Pithing is a quick method of brain destruction achieved by insertion of a needle through the foramen magnum.

Q.4.2.4

Recommended Methods for Euthanasia for Specific Animal Species

Mouse

- Decapitation
- Cervical dislocation with subsequent exsanguination
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rat

- Concussion, cervical dislocation (both with subsequent exsanguination), and decapitation, conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 150 mg/kg i.p.
- Microwave irradiation

Hamster

- Decapitation; conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 300 mg/kg i.p.

Guinea Pig

- Concussion (with subsequent exsanguination), and decapitation; conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rabbit

- Stunning with captive bolt
- Concussion (with subsequent exsanguination); conducted only by well trained persons
- Sodium pentobarbitone at a dose of 120 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter

Cat

- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 200 mg/kg i.p.

- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter; it is recommended to anesthetize the animal beforehand with 20–30 mg/kg ketamine i.m. or 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Dog

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v., it is recommended to anesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Ferret

- Sodium pentobarbitone at a dose of 120 mg/kg i.p.

Cattle, Sheep, Goat, Horse, Pig

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; for larger animals prior sedation is recommended (Xylazin).
- All other acceptable methods which are used for slaughtering

Primate

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; it is recommended to anaesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.
- T61 at a dose of 0.3 ml/kg strictly i.v., it is recommended to anesthetize the animal beforehand with xylazine/ketamine i.m.

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